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	Page
<i>Madsen, Valdemar, Pedersen-Bjergaard, K., Roholt, K. and Tønnesen, M.</i> : The antigen and pyrogen content in commercial chorionic gonadotrophin preparations, with reference to their clinical application	140
<i>Ostergaard, Erling and Hamburger, Christian</i> : The formation of antihormones in women treated with pregnant mares' serum hormone (Antex)	148
<i>Wahlberg, Johannes</i> : The thiouracil derivatives and their use ..	165
<i>Tärnblom, Nils</i> : Renal crises following the extirpation of parathyroid adenomata	178
Finnish Society for Endocrinology	198

FASC. III. JULY

<i>Fernández-Morón, H. and Luft, R.</i> : Submicroscopic cytoplasmic granules in the anterior lobe cells of the rat hypophysis as revealed by electron microscopy	199
<i>Bruun, Anton F., Hemmingsen, Arel M. and Møller-Christensen, E.</i> : Attempts to induce experimentally maturation of the gonads of the European eel, <i>Anguilla anguilla</i> L	212
<i>Kroon, D. B.</i> : The effect of the hypofunction of the thyroid gland, induced by methylthiouracil, on the phosphatase activity of some organs and on the process of ossification in the rat and in the guinea-pig	227
<i>Claesson, L., Hillarp, Nils-Åke, Högberg, B. and Hökfelt, B.</i> : Changes in the ascorbic acid content in the interstitial gland of the rabbit ovary following gonadotrophic stimulation	249
<i>Hamburger, Chr. and Kaae, S.</i> : Testosterone treatment and 17-ketosteroid excretion. Investigations on the influence of the mode of administration upon the absorption and excretion of testosterone propionate	257
<i>Luft, R. and Sjögren, B.</i> : The effect of desoxycorticosterone acetate (DCA) and sodium chloride on blood pressure in postural hypotension and arterial orthostatic anemia	287
Swedish Society for Endocrinology	306
Danish Society for Endocrinology	306

FASC. IV. SEPTEMBER

<i>Lederer, J.</i> : Association du polyvinyl-pyrrolidone à la pituitrine, dans le traitement du diabète insipide	307
<i>Gaarenstroom, J. H., Hublé, J. and de Jongh, S. E.</i> : Growth hormone and blood sugar level	317
<i>Ljung, Olof</i> : The electrocardiogram in hypocalcemia and hypothyreosis	324

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INVESTIGATIONS INTO THE QUANTITATIVE DETERMINATION OF ANTIHORMONES AGAINST PREGNANT MARES' SERUM HORMONE*)

BY

CHRISTIAN HAMBURGER and ERLING ØSTERGAARD

It is a well-known fact that certain hormones can give rise to the formation of inhibitory substances in the blood. The conditions necessary for the formation of these »anti-hormones« are, 1° that the hormones originate from a species other than that of the experimental animal, 2° that they are related to the proteins, and 3° that they are given for a sufficient length of time. The most important antihormones are those which antagonise the action of gonadotrophins and thyrotrophin.

Since the formation of antihormones active against pregnant mares' serum gonadotrophin plays an important role in the therapeutic action of this substance, the determination of inhibitory substances in the blood serum is of practical value. The comparison of the inhibitory activity of various serum specimens is obviously desirable, and hence the question of a quantitative assay is of great significance.

The presence of antigonadotrophic substances in the serum

*) Paper given at the meeting of the Scandinavian Societies for Endocrinology in Stockholm, September 25—26, 1948.

is usually determined as follows: one group of animals, preferably immature female rats or mice, are injected simultaneously with gonadotrophin and the serum specimen, and another group with the hormone alone. The decrease in the ovarian or uterine weights of the serum-treated animals, as compared with those injected with gonadotrophin alone, or the morphological appearance of the ovaries, serve as criteria of the antigonadotrophic action of the serum. The hormone preparation and the serum are injected separately or after mixing. The assay can be performed in several ways: The animals receive a constant dose of gonadotrophin and different amounts of serum, and the smallest amount of serum which completely inhibits the action of the hormone is determined. Or, the serum dose may be kept constant and the amount of hormone varied; the largest amount of gonadotrophin which is completely inhibited is then determined. The activity of the antiserum can also be calculated from the results of experiments in which only a partial inhibition of a certain dose of gonadotrophin was obtained, provided that dose-response curves are available.

Most investigators have measured the activity of antigonadotrophic sera using the above-mentioned methods (*Rowlands & Spence, 1939, Zondek & Sulman, 1942, and Østergaard, 1942*; further references are found in these publications). The validity of the determination of an antigonadotrophic »titer« of serum specimen is, however, dependent upon the following assumption: it presupposes that when 0.1 ml. of serum is found to be the smallest dose which neutralizes 1 mg. of a gonadotrophic preparation, one millilitre should be the smallest dose which neutralizes 10 mg. of this gonadotrophin. The truth of this assumption has, to our knowledge, never been proved, and it is the aim of the present investigation to throw light on this problem.

MATERIAL AND TECHNIQUE

All the experiments described in the present communication were carried out with a single sample of mares' serum

gonadotrophin and a single specimen of antigonadotrophic serum. The *hormone* used was a commercial preparation, Antex Leo*) No. 460910, which, diluted with lactose, had an activity of 12.5 I. U. per milligram. The *antiserum* (No. 368/47) was a pooled specimen from 4 adult rabbits treated for two months with daily subcutaneous injections (except on sundays) of 10 mg. of *Antex F 368*, the total dose being about 2400 I. U. The serum was stored in small vials in the refrigerator at — 11 to — 20° C. Preliminary experiments with several other pregnant mares' serum gonadotrophins and other antisera have given results similar to those reported here. The *experimental animals* were immature female rats from the breeding colony of the State Serum Institute; they were used when 26 to 28 days old. The animals received 5 subcutaneous injections of mixtures of hormone and serum or dilutions thereof over a period of 48 hours. Uterus and ovaries were weighed at autopsy about 96 hours after the first injection. The present experiments were performed on 574 rats, injected with hormone and serum, and 215 rats, used for the determination of the dose-response curves for the *Antex* preparation.

RESULTS

The dose-response curves (uterine and ovarian weight in rats) for *Antex* No. 460910 are shown in Fig. 1. From these curves it is possible to calculate the amount of gonadotrophin to which the uterine and/or ovarian weights in the animals treated with hormone + serum correspond. An antihormone assay with constant hormone dose and different amounts of antiserum is shown in Fig. 2. In this and the following diagrams the interrupted lines indicate the uterine and ovarian weights for the hormone alone, the white and black circles represent the uterine and ovarian weights actually found in the animals treated with hormone + serum. The average values are obtained from at least 3 and at most 33 rats. From

*) The authors were kindly supplied with the *Antex* preparations by *Lovens kemiske Fabrik*, Copenhagen.

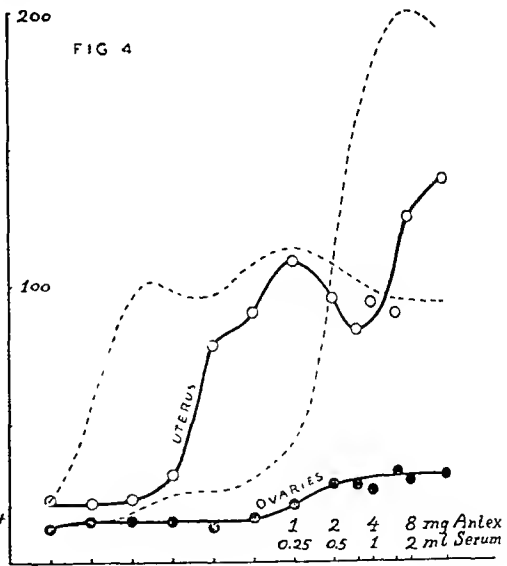
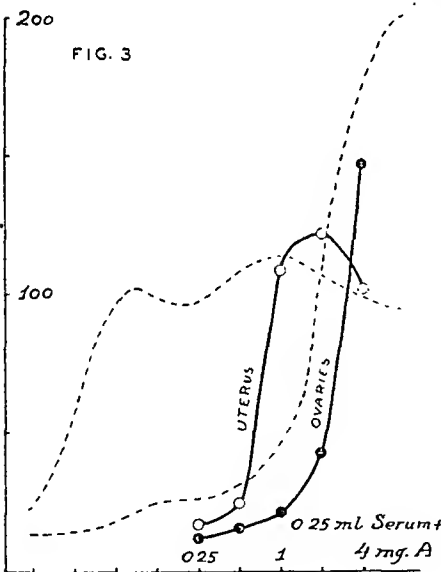
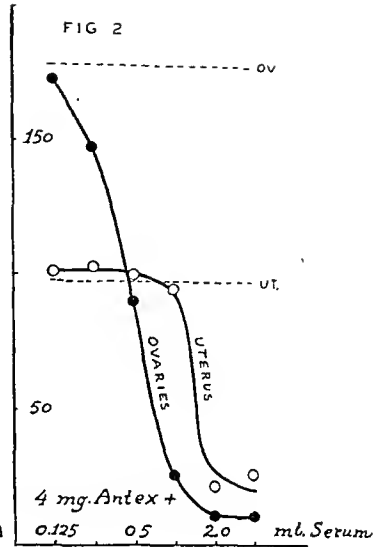
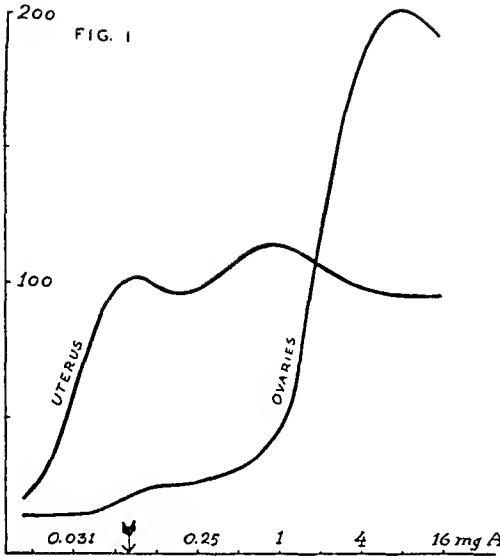


Fig. 1.

Uterine and ovarian dose-response curves for the pregnant mares' serum gonadotrophin *Antex* Leo No. 460910. The arrow indicates the position for 1 I. U. of the preparation. Ordinate: Organ weight in mg. Abscissa: Dose of gonadotrophin, log. scale. Test animals: immature rats.

Fig. 2.

Uterine and ovarian weight curves obtained in rats treated with a constant amount of *Antex* and different quantities of antiserum. White circles: average uterine weights. Black circles: average ovarian weights. Interrupted lines: organ weights obtained with the hormone alone.

Fig. 3.

Uterine and ovarian weight curves obtained in rats treated with different amounts of *Antex* and a constant dose of antiserum. (See also legends to preceding figures.)

Fig. 4.

Uterine and ovarian weight curves obtained in rats treated with *Antex* and antiserum in constant proportions. (See also legends to Fig. 1 and 2.)

Fig. 2 it can be seen that 0.125 ml. of serum has no significant effect on the organ weights. 0.25 ml. decreased the ovarian weight to 148 mg. which, according to the dose-response curve, is equal to 2.6 mg. of the *Antex* preparation, i. e. this serum dose has inhibited 1.4 mg. of the 4.0 mg. of *Antex*. One ml. therefore, ought to neutralize 5.6 mg. of the preparation but actually this dose did not inhibit the 4.0 mg. given. Complete inhibition does not occur until the dose of serum is increased to 2 ml. There is thus no simple relation between dose and effect produced. This fact is also evident from an experiment in which the rats received a constant amount of antiserum and different doses of hormone (Fig. 3), since 0.25 ml. of serum neutralized 1.4 mg. when mixed with 4.0 mg. of *Antex*, though the same dose of serum did not completely inhibit 1.0 mg.

Fig. 4 gives the results of another experiment in which the ratio between the amounts of hormone and serum was constant at all dose levels. If there existed any relation between dose and effect the dose-response curves should be identical with those for the hormone alone, the curves being merely shifted horizontally to the right. The shape of the curves, however, is appreciably altered; this is especially the case for the ovarian weights of the rats treated with 6, 8 and 16 mg. which are disproportionally low.

The average ovarian and uterine weights found at each of the combinations of *Antex* and antiserum in the whole investigation, together with the number of rats used, are given in Table 1. By means of the dose-response curves it is possible, for each combination, to calculate the effective amount of hormone left in the mixture. By subtracting this value from the dose of hormone injected, the amount of gonadotrophin inhibited by the dose of serum can be found. If the »titer« of an antigonadotrophic serum is defined as *the reciprocal value of the smallest amount of serum, measured in ml., which inhibits 1 I. U. of the gonadotrophin* (e. g. the »titer« is 32 when 1/32 ml. serum neutralizes 1 I. U.) we arrive at the values given in Table 2. This table does not include all the data given in Table 1, some of which have been omitted for various reasons: Firstly, when the inhibition is complete we can only obtain a minimal value for the »titer« (e. g. ≥ 64); secondly, the calculation has not been recorded in cases where the serum dose is exceedingly low in comparison with the amount of gonadotrophin, because the experimental error is too high to allow of an estimate of the »titer«. From Table 2 it is seen that the »titer« varies from 25 to 274; but the high and low figures are, however, not spread out irregularly, but are, on the contrary, arranged in a quite systematic manner, the values increasing from left to right and from above downwards. The exceptions from this rule may reasonably be attributed to experimental error. The distribution of the »titer«-values indicates that the small antiserum doses are relatively much more effective than larger ones.

Table 1.

Average uterine and ovarian weights of immature rats treated with mixtures of pregnant mares' serum gonadotrophin and rabbit anti-gonadotrophic serum in various combinations. (Roman figures: uterine weight; figures in italics: ovarian weight; figures in brackets: number of rats.)

		Gonadotrophin (Antex 460910, 1 mg. = 12.5 I. U.) (Dose in mg.)																
		1/64	1/32	1/16	1/10	1/8	1/4	1/2	1	3/2	2	3	4	6	8	16		
Antiserum 308/47 (Dose in mL)	4												27 11 (5)		54 13 (3)	111 32 (3)		
	2												22 11 (5)		127 30 (10)			
	3/2													91 33 (5)				
	1										28 13 (3)		95 26 (26)		92 120 (4)			
	3/4									22 16 (6)		85 28 (6)						
	1/2							30 16 (5)	22 12 (5)	81 25 (6)	97 29 (11)		100 90 (17)					
	1/2.7									118 22 (6)								
	1/3.3									90 32 (6)								
	1/4						17 12 (6)	25 16 (16)	110 21 (21)	105 32 (6)	121 43 (22)		103 148 (33)					
	1/8					25 14 (5)	27 14 (5)	91 16 (5)	90 31 (6)		121 64 (6)		101 173 (5)					
	1/16		21 16 (5)		26 16 (5)	83 13 (5)	120 31 (5)	121 35 (6)		130 66 (5)								
	1/20			22 15 (6)														
	1/25			21 17 (5)														
	1/32		27 13 (5)	27 15 (17)	32 15 (5)	106 19 (11)	107 26 (5)	141 34 (5)		111 71 (5)								
	1/40			25 15 (17)														
	1/50			40 17 (24)														
1/64		27 15 (5)	21 15 (5)	51 16 (17)	101 16 (11)	103 18 (5)	102 29 (5)	110 43 (5)										
1/128	20 11 (5)	21 14 (5)	35 14 (11)		97 14 (5)	111 21 (5)	122 25 (4)	121 48 (5)										
1/256	23 11 (5)	30 14 (11)	67 17 (5)		91 19 (5)	110 24 (5)	108 22 (5)	117 53 (5)										
1/512	24 15 (6)	29 12 (5)	47 11 (5)		89 14 (5)	83 23 (4)	94 21 (5)	127 50 (5)										

Table 2.
Gonadotrophin (Antex 460910, 1 mg. = 12.5 I. U.)
(Dose in mg.)

		1/32	1/16	1/8	1/4	1/2	1	2	4	8	16
Antiserum 368/47 (Dose in ml.)	4									25	48
	2									47	
	1								48	74	
	1/2							40	58		
	1/4						45	50	70		
	1/8					44	50	60	50		
	1/16				40	100	66	120			
	1/32			42	72	120	152				
	1/64			60	154	80					
	1/128		64	104	274						
	1/256	100	72	164							
	1/512	200	220								

Calculation of the »titer« of the antiserum in various combinations of serum and gonadotrophin. The figures indicate the number of I. U. of the gonadotrophin neutralized by 1 ml. of serum, as calculated from the dose-response curves.

DISCUSSION

The experiments described above were all carried out with a single preparation of pregnant mares' serum gonadotrophin and a single rabbit antigonadotrophic serum. The conclusions drawn from these investigations are, however, strongly supported by experiments with several other *Antex* samples and other rabbit antisera.

The activity of the antigonadotrophic serum was tested in immature female rats, which were injected subcutaneously with mixtures of hormone and antiserum. No objection can be raised to the use of the mixture procedure when dealing with gonadotrophin from pregnant mares' serum, since the effectiveness of this hormone is quite independent of the rate of absorption (*Hamburger & Pedersen-Bjergaard, 1938*).

Our experiments have shown that the antigonadotrophic activity of a serum depends on the ratio between the amount of serum and that of gonadotrophin. The statement that a certain amount of serum neutralizes a certain amount of hormone does not allow of any conclusion as to the amount of hormone which is inhibited by any other dose of serum. The smaller doses of serum inhibit relatively much more gonadotrophin than the higher doses. The value of »antigonadotrophic units« or of an antiserum's »titer« (for references, see *Zondek & Sullman*, 1942, and *Østergaard*, 1942) is, therefore, rather problematic, and calculations of the quantity of gonadotrophin expected to be completely neutralized by the total volume of the circulating blood serum (*Rowlands & Spence*, 1939) must be regarded as quite useless.

A rough estimate of the activity of an antigonadotrophic serum may be obtained when the conditions for the assay are strictly standardized. As a matter of fact these requirements have been fulfilled by a considerable number of the investigators as judged from the world literature. It is interesting to note that, when trying to elucidate the quantitative laws of hormone-antihormone reactions, we encounter the same difficulties as those found for toxin-antitoxin reactions. This fact supports the view that the problems of antihormones belong to immunology.

SUMMARY

A total number of 574 immature female rats were injected subcutaneously with mixtures of a commercial preparation of pregnant mares' serum gonadotrophin and an antigonadotrophic serum from rabbits treated for two months with another sample of the hormone.

The uterine and ovarian weights obtained in rats treated with various combinations of hormone and antiserum were compared with the corresponding dose-response curves obtained in animals treated with pregnant mares' serum gonadotrophin alone. From these curves the amount of hormone neutralized by the antiserum was calculated.

The small doses of serum were found to be relatively much more effective than larger quantities, and the antigonadotrophic »titer« of the serum could therefore, with equal justification be calculated as any value between 25 and 274. Since the laws governing the reaction between gonadotrophin and antigonadotrophin are as yet unknown, any statement concerning the »titer« of an antiserum is of little value.

A rough estimate of the activity of an antigonadotrophic serum may be obtained, when the conditions of assay are strictly standardized.

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STUDIES ON THE LOCALIZATION OF HYPOTHALAMIC CENTRES CONTROLLING THE GONADOTROPHIC FUNCTION OF THE HYPOPHYSIS*)

From the Department of Histology,
University of Lund. (Professor G. Glimstedt, M. D.)

BY

NILS-ÅKE HILLARP

Several investigations, especially those performed by *Ranson, Dey*, and co-workers (*Dey, Fisher, Berry & Ranson*, 1940, *Dey*, 1941, 1943, *Brookhart, Dey & Ranson*, 1941, *Dey, Leininger & Ranson*, 1942, and *Alphin & Dey*, 1944) have demonstrated the existence in the hypothalamus of certain centres controlling the gonadotrophic function of the hypophysis. Large lesions, made in the appropriate place in the anterior hypothalamus in guinea-pigs, cause a marked decrease of the secretion of the luteinizing hormone (LH), while damage to the median eminence, largely inhibits the secretion of both follicle stimulating hormone (FSH) and LH, with consequent atrophy of the genital organs. These hypothalamic centres have not been accurately localized, possibly because of the difficulty of producing small limited lesions in exact bilateral symmetry. The present investigation is an attempt to localize

*) Aided by a grant from the Faculty of Medicine, University of Lund.

these centres by means of a new instrument capable of producing brain lesions in rats.

MATERIAL AND METHODS

The experiments were carried out on adult female albino rats, inbred for eight generations.

The instrument used to produce brain lesions has been described in a previous report (*Hillarp, 1947*). As a rule only animals weighing between 170 and 200 gm. were used, so that the material should be as homogeneous as possible. With female rats of this weight, preliminary experiments determining the coordinates of the various hypothalamic nuclei were performed. In the main experiments (the operation is best performed under ether anaesthesia and not — as previously stated — under nembutal anaesthesia), only one lesion was usually made on each side of the third ventricle, but sometimes two were made. The position of the lesions was varied in antero-posterior direction from the optic chiasma to the mammillary body. The distance between the two lesions varied between 1 and 2.5 mm. The level was determined by lowering the electrode (by its own weight) to the base of the skull and subsequently raising it (0—1 mm.) to the desired position. — The lesions were produced by means of a current of 0.3—0.5 mA for 15 to 30 seconds.

Three to five weeks after the operation the vaginal cycle was studied by means of daily vaginal smears for a period of 2—3 weeks. Moreover, in those animals which could be mated, pregnancy, parturition, and lactation were observed. — The urinary output was measured in a metabolism cage starting at the earliest one month after operation. None of the animals had diabetes insipidus.

The animals were killed 1½—4 months after the operation. The hypothalamic region was fixed in Carnoy and cut in serial sections of 10 μ . Every fourth section was stained in gallo-cyanin according to *Einarson* or in cresyl violet. The ovaries, adrenal glands, and thyroid glands were fixed in 10 per cent

formalin, cut in serial sections of 10 μ , and stained in hematoxylin-eosin. The hypophyses were fixed in Susa and stained in Azan (serial sections of 5 μ).

RESULTS

Controls.

The experiments were done on 149 rats. Fiftytwo of these showed no direct damage in the hypothalamus other than the perforation made by the electrode.¹⁾ These animals, therefore, were used as controls in order to determine the extent of the spontaneous disturbances in the sexual functions occurring in the material. Serious disturbances, however, were only observed in one animal which had an irregular cycle (long periods of dioestrus), and did not become pregnant in spite of being kept with a male for a long period. Two more animals had irregular cycles with long dioestrus intervals but both became pregnant. One animal refused to take care of her young after parturition. Otherwise the rats exhibited normal sexual functions in so far as these could be determined by the methods used.

Rats with constant oestrus.

In 29 of the animals with injuries within the hypothalamus the vaginal cycle was discontinued and a state of practically constant oestrus ensued. The histological examination of the ovaries revealed the presence of both large and small follicles but a complete absence of corpora lutea (Fig. 1) (one animal had a few very small corpora lutea). These animals thus show the same genital changes as were found by *Dey* in guinea-pigs with large lesions in the anterior hypothalamus.

Thus the ovaries in this group of rats continuously pro-

1) This is due to the fact that in a great number of cases, in order to minimize the injury as much as possible, the electrode was uninsulated only at the tip so that if the electrode was insufficiently raised above the base of the skull, the trauma was produced below the hypothalamus.

duced oestrogenic hormone. This is also proved by the fact that the uterus was markedly dilated and thick-walled in the animals which lived for several months after the operation. In order to obtain an idea of the amount of this secretion,



Fig. 1.

General picture of the ovary of a rat with constant oestrus after hypothalamic lesions (See Figs. 6—8). Follicles of various size but not corpora lutea are present. Htx-eosin. $\times 40$.

the occurrence of oestrogen-precursor in the interstitial gland was examined in one ovary (using the methods described by *Claesson & Hillarp, 1947*). This showed that in all the ovaries the whole gland was abundantly filled with precursor. This finding strongly supports the assumption that the oestrogen secretion remains at a fairly low level and that in any case no excessive production occurs. During the rats' normal oestrous phase the precursor content is reduced to scarcely demonstrable amounts (*Claesson & Hillarp, 1947*).

In addition to the absence of corpora lutea the ovaries also contain an extremely well developed interstitial gland (Fig. 2),

probably resulting from the proliferation of the theca cells around degenerated follicles, and consisting of large, light cells, rich in protoplasm and with normal nuclear appearance.

This endocrine disturbance can be consistently produced by placing relatively large basal bilaterally symmetrical in-



Fig. 2.

Larger magnification of the same ovary as in Fig. 1. Abundant interstitial gland with large cells rich in protoplasm. Htx-eosin. $\times 90$.

juries immediately lateral to the ventricular wall below, or just caudal to, the paraventricular nucleus. A closer study of the localization of the lesions in animals both with and without constant oestrus gives the following result: Injuries — even large ones — in the anterior hypothalamus but ending anterior to the paraventricular nucleus, as well as injuries situated in the posterior hypothalamic area caudal to the hypophysial stalk, do not produce any changes in the ovaries (in certain cases, however, there are disturbances in the sexual cycle, see below). On the other hand, lesions stretching from the anterior hypothalamic area to the paraventricular nucleus, damaging the largest part of the caudal portion of the anterior

hypothalamic area down to the chiasma, and lateral to the medial forebrain bundle, produce continuous oestrus. It was not possible to produce this disturbance with *small* injuries within the area anterior to the paraventricular nucleus. But

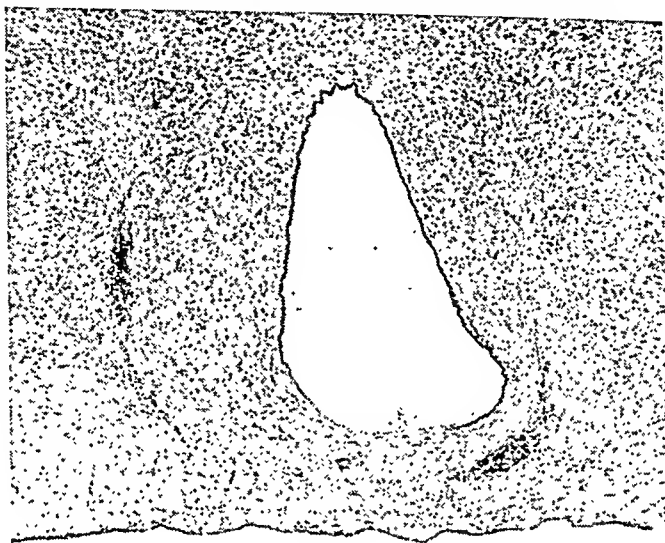


Fig. 3.

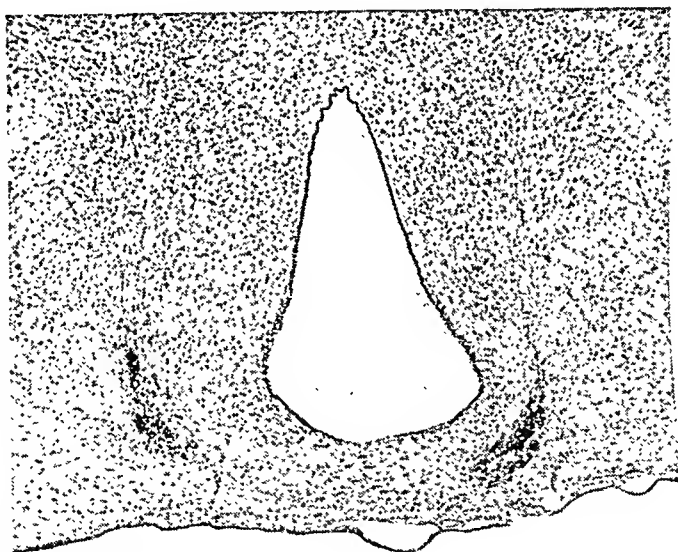


Fig. 4.

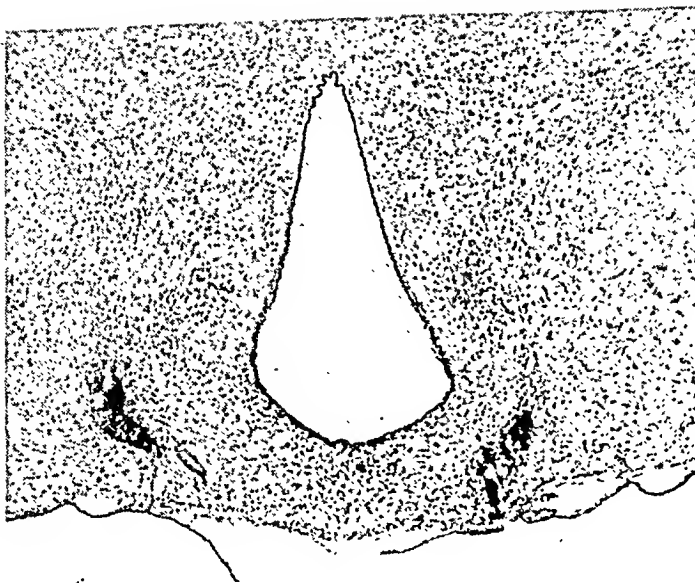


Fig. 5.

Figs. 3—5. Position and extent of the lesions in a rat with constant oestrus. Small, basal injuries on both sides of the 3rd ventricle between the paraventricular nucleus and the anterior border of the median eminence. Fig. 3 shows a section cut immediately caudal to the paraventricular nucleus, Figs. 4 and 5 sections 120 and 240 μ respectively caudal to the first one. The lesions appear asymmetrical because of the dilatation of the 3rd ventricle to the right, and because of a certain oblique sectioning of the preparation. Sections of 10 μ . Cresyl violet, $\times 40$.

within the area between the paraventricular nucleus and the hypophysial stalk, even very limited lesions may bring about the typical changes in the ovaries. The lesions must be basal, perfectly bilaterally symmetrical, and situated approximately between the 3rd ventricle and the lateral hypothalamus (the position can best be seen in Figs. 3—8). More dorsal or lateral injuries do not seem to have any effect on the sexual functions. — The following nuclei are generally undamaged in animals with constant oestrus: the supraoptic, suprachiasmatic, paraventricular, ventro- and dorso-medial, and arcuate nuclei as well as the lateral hypothalamic area.

The findings in the controls as well as the fact that none

of the 23 animals with asymmetrical lesions within the hypothalamus showed constant oestrus, make it evident that the disturbance in these animals did not occur spontaneously.

No difference was observed in the adrenal and thyroid glands of the animals with constant oestrus and those of the

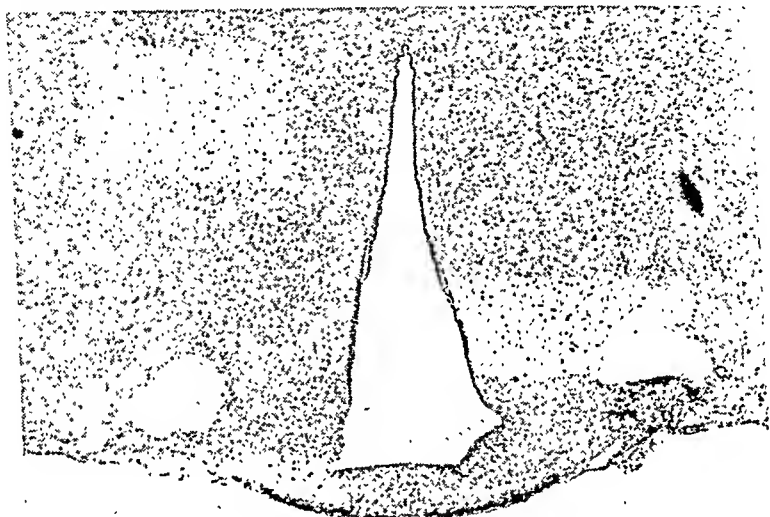


Fig. 6.

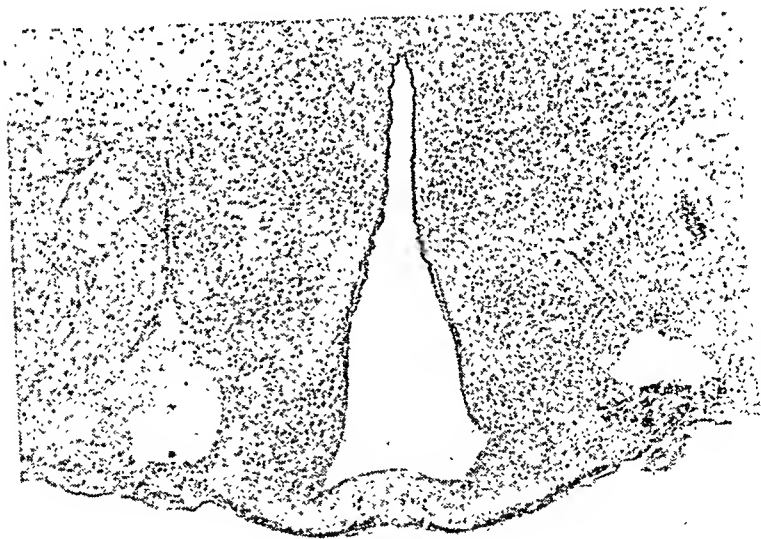


Fig. 7.

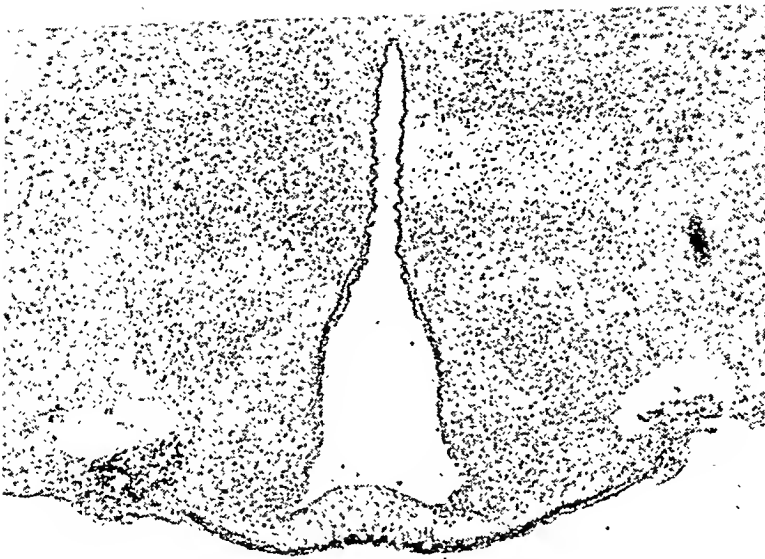


Fig. 8.

Figs. 6—8. Position and extent of the lesions in a rat with constant oestrus (sections of this animal's ovaries are shown in Figs. 1 and 2). Small basal injuries on both side of the median eminence approximately midway between the anterior border of the eminence and the hypophysial stalk (interval between each section 120 μ). Sections of 10 μ Cresyl violet. $\times 40$.

control animals. The hypophyses in the rats with large lesions in the anterior hypothalamus showed a distinctly larger number of α -cells than did the normal pituitaries. As a matter of fact *Alphin & Dey* drew attention to this as early as 1944. However, the rats in which a constant oestrus had been induced by means of small basal injuries caudal to the paraventricular nucleus, did not show any such change. The hypophysis in this group showed an apparently normal cellular picture. The increase in the number of α -cells, therefore, can not be directly connected with the change in the LH-secretion of the hypophysis.

Rats with other disturbances in the sexual functions.

In 11 rats with various types of injuries — generally rather small — in the anterior hypothalamus anterior to the paraventricular nucleus, an irregular vaginal cycle was observed

with long periods of dioestrus, alternating with short periods of oestrus. These animals did not become pregnant although they were kept in cages with male animals for varying periods. The ovaries showed no demonstrable changes: both the follicles and the corpora lutea seemed intact.

In another 7 rats with lesions in the same area, disturbances of pregnancy or parturition occurred. The number of still born foetuses was unusually large (50—100 per cent) in some animals, in others the foetus or the placenta was retained.

Since this group of rats is small, and many changes rather difficult to evaluate exactly were observed in them, no attempt was made to localise the injuries more accurately.

DISCUSSION

The phenomenon of constant oestrus and the typical ovarian changes, here described in rats, have been carefully investigated by *Ranson, Dey* and co-workers after injuries of the hypothalamus, in guinea-pigs. Their investigations show unmistakably that the disturbances of the ovarian function are due to a change in the gonadotrophic secretion of the hypophysis. An obvious assumption is that the secretion of LH is inhibited. *Dey* (1943 a) has supported this assumption experimentally by demonstrating that copper acetate does not — as in normal animals — produce ovulation in guinea-pigs with constant oestrus. However, the LH-production of the hypophysis is not entirely inhibited, since the ovaries show a continuous secretion of oestrogen. This secretion is only possible with the cooperation of LH (for references, see *Fevold*, 1944). The fact that the interstitial gland has cells rich in protoplasm and with normal cellular structure also proves that not only FSH is secreted from the hypophysis, as this hormone cannot repair the atrophy of the interstitial cells following the disappearance of LH (*Fraenkel-Conrat, Simpson & Evans*, 1940, *Fraenkel-Conrat, Li, Simpson & Evans*, 1940). At the present stage of the investigation it may be said that specifically localized injuries to the hypothalamus destroy centres that control the LH-secretion of the hypophysis, or cut off the

connections between these centres and the hypophysis, the result being a reduction — but not total cessation — of the LH-secretion.

In their studies on the role of the hypothalamus in the sexual functions *Ranson, Dey*, and co-workers have produced such large lesions (3 injuries: one in the middle line and one on each side of this line; current of 3 mA for 30 seconds) that no exact localization of gonadotrophic secretion centres was possible. It is evident from the present investigation that a centre for LH-secretion must exist in the anterior hypothalamus, immediately anterior and ventral to the paraventricular nucleus. In this area the lesions have to be fairly large in order to produce a state of constant oestrus in the animals, and it also seems to be essential that they should be localized in the caudal portions of the anterior hypothalamic area. The fact that the same disturbances can be produced by very small basal injuries caudal to the paraventricular nucleus indicates the existence of a fairly well defined fibre system passing from the anterior hypothalamic area, and running superficially on both sides of the median eminence down towards the hypophysial stalk. These results agree very well with those obtained by *Clark* (1942) in an attempt to localize a sexual centre in the hypothalamus. He made transverse lesions extending from fornix to fornix at various levels, and found that such lesions did not necessarily disturb the normal sexual behaviour. *Clark*, therefore, thought it improbable that a sexual centre was present in the anterior hypothalamus. However, he admits the possibility that a superficial fibre system may exist which is not destroyed by such lesions. The present investigation demonstrates the presence of such a fibre system, and thus explains why *Clark* arrived at results quite opposite to those obtained by *Ranson, Dey*, and co-workers. — The occurrence of disturbances in the vaginal cycle, pregnancy, and parturition after small injuries within this region, further supports the view that a centre for the gonadotrophic functions of the hypophysis is situated in the anterior hypothalamic area.

In this connection it should also be pointed out that *Dey*

found in his investigations (1941, 1943 b) that the lesions in the median eminence which destroy the connections between the hypothalamus and the hypophysis, produce a marked genital atrophy in guinea-pigs. Similar changes too were demonstrated by *Westman* and co-workers in rats and rabbits after severing the hypophysial stalk (*Westman & Jacobsohn*, 1937, 1938, 1940, 1942, *Westman, Jacobsohn & Hillarp*, 1943, *Hillarp & Jacobsohn*, 1943). The results of these latter investigations are thus strikingly confirmed by *Dey*. The significance of this work has not been sufficiently appreciated, and the discussions of this subject are still too much influenced by the conclusions of *Dempsey* (1939), *Dempsey & Uotila* (1940), and *Uotila* (1940), who deny the existence of hypothalamic centres controlling the gonadotrophic function of the hypophysis through the hypophysial stalk.

SUMMARY

Investigations on the localization of the centres controlling the gonadotrophic function of the hypophysis were carried out on female rats. Brain lesions of different sizes were made with a new instrument in various regions of the hypothalamus.

It appears from these experiments that a centre controlling the LH-secretion of the hypophysis must exist in the anterior hypothalamic area immediately anterior and ventral to the paraventricular nucleus. Comparatively large lesions within this area produce typical ovarian changes (the occurrence of follicles and an abundant interstitial gland but no corpora lutea) and a state of constant oestrus in the animals. The essential factor seems to be that the lesions should be confined to the caudal portions of the anterior hypothalamic area. Caudal to the paraventricular nucleus the same disturbances can be produced by means of very small and bilaterally symmetrical basal injuries, thus indicating that a fairly well demarcated fibre system originates in the anterior hypothalamic area and runs superficially on both sides of the median eminence down towards the hypophysial stalk. The

destruction of this centre or of its fibre system does not, however, cause a total inhibition of the LH-secretion.

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STUDIES ON THE STORAGE MECHANISM OF THE OESTROGEN-PRECURSOR*)

BY

BERTIL ALDMAN, LENNART CLAEßSON, NILS-ÅKE HILLARP
and ERIK ODEBLAD

The occurrence in the ovary of a precursor of the oestrogenic substances which can be demonstrated by histochemical methods is of value in the study of the formation of these substances. In order to obtain a firmer basis for the analysis of the formation of oestrogenic hormone the conditions under which the precursor is stored and transformed into a hormone must be determined. In the present paper some investigations on this problem are reported.

MATERIAL AND METHODS

In these experiments only adult female albino rats, inbred for eight generations, were used.

Hypophysectomy was performed by a parapharyngeal approach, the completeness of the removal being checked by means of serial sections of the operated area.

The pregnant mares' serum gonadotrophin *Antex Leo* (PMS) was used, as well as an electrophoretically homogeneous crystalline chorionic gonadotrophin (*Gonadex Leo*; for

*) The investigations were carried out with support from Statens Medicinska Forskningsråd.

further information about this hormone, see *Claesson, Högborg, Rosenberg & Westman, 1948*).¹⁾ The injections were given subcutaneously.

The vaginal reaction was controlled by daily vaginal smears.

The histochemical and polarizing optical studies of the ovaries were carried out in the same way as in previous investigations (*Claesson & Hillarp, 1947*). In addition, the ovaries were examined histologically after staining with hematoxylin and eosin.

RESULTS

The fact that the ovaries of immature and anoestrous rabbits contain little or no precursor suggests that the storage of the precursor in the interstitial glandular cells is dependent on a hypophyseal stimulus. It is therefore quite conceivable that the storage mechanism is controlled by one of the two gonadotrophic pituitary fractions, the follicle stimulating hormone (FSH) and the luteinizing hormone (LH). An examination of the precursor after administration of FSH or LH should give an answer to this question. As these fractions were, however, not available in a pure state it became necessary to try a more indirect method and use pregnant mare serum gonadotrophin (PMS) and chorionic gonadotrophin (PU).

All the experiments were carried out on hypophysectomized animals in order to eliminate any effect arising from the animal's own gonadotrophic hormones.

In an initial series, (13 animals), the precursor formation was studied only after hypophysectomy. The operation was performed in the dioestrous, pro-oestrous and oestrous phases of the vaginal cycle. One ovary was removed 1 day after, and the other 8 days after the hypophysectomy. All the ovaries

1) Leo and Co., Hälsingborg, have kindly assisted us in our investigations by placing the preparations at our disposal.

contained large stores of the precursor. *The precursor thus remains at a high level after hypophysectomy, independently of the particular sexual phase present when the operation is performed.*

The high precursor content in the ovaries of the hypophysectomized animal might perhaps be interpreted as the result of an autonomic storage process in the interstitial cells independent of a gonadotrophic hormone stimulus. In order to find out whether such an autonomous mechanism exists the precursor present in the ovaries must first be used up (*»mobilised«*). In a series of 7 animals, therefore, 100 IU Antex was injected 5 days after hypophysectomy. One ovary in each animal was removed 2 days after the injection as a control of the mobilization, and the other 7 days later. All parts of the control ovaries showed an almost complete absence of the precursor. The ovaries removed at the later date, however, also had a very low precursor content. *Hence an acute mobilization of the precursor, produced by a high dose of Antex in hypophysectomized rats, is not followed by storage of the precursor in the interstitial gland.*

It appears from this experiment that the storage cannot be entirely an autonomous cellular process, but is most probably controlled by a gonadotrophic hormone. In order to confirm this, the experiment was repeated with the modification that a dose of 1/3 IU Antex or 1/30 γ electrophoretically homogeneous PU was injected daily, during the 7 days following the removal of the first ovary (5 animals in the first series, 4 animals in the second). This dose proved sufficiently large to produce vaginal oestrus without, however, giving a clear uterine reaction. On the other hand it did not entirely prevent the atrophy of the interstitial gland which, even after this treatment, contained densely packed cells poor in protoplasm. The ovaries removed first, contained practically no precursor, but those removed at a later date, however, contained large amounts of it. *Hence in hypophysectomized rats the administration of small doses of Antex or highly purified PU pro-*

duces a marked storage of precursor in the interstitial gland in ovaries where the precursor has been previously mobilized by a large dose of Antex.

It may seem strange that boths PMS and PU have the same effect on the storage of the precursor. The PMS-preparation used, however, probably contains small amounts of an LH-factor (this question, and the relationship between PMS and PU on the one hand and FSH and LH on the other, are dealt with in the discussion), of which the known biological effects on the ovary are similar to — and probably identical with — those of PU. It is therefore possible that the storage of the precursor when 1/3 IU Antex is given daily, is due to this LH-factor. In order to clear up this problem the experiment had to be repeated with a purer PMS preparation. Taking advantage of the fact that injected PU or hypophysial LH are rapidly eliminated from the blood, while the elimination of PMS occurs slowly, (cf., Zondek & Sulman, 1945), Antex was purified in the following manner:

A female rabbit (weight: 3 kg.) was castrated and hypophysectomized and three weeks after the operation 48.000 IU Antex was injected intravenously. Four days later the animal was bled to death under ether anaesthesia. The total amount of serum collected was precipitated with 5—6 volumes of cold alcohol and the precipitate was washed and dried with acetone. The dry powder was stored in vacuo in the refrigerator. When tested on immature mice this serum powder was found to contain approximately 1 IU PMS per 1.3 mg. — If a *contaminating* LH-factor is present in Antex, this factor must have almost entirely disappeared from the blood four days after the injection. The testing of the biological purity of the preparation will be reported in a later paper.

This purified PMS was used in an experiment similar to those described above. Five days after hypophysectomy, 100 IU Antex was injected in 4 female rats in order to mobilize the precursor. One ovary was removed three days later as a control of the mobilization, and 0.4 mg. of the prepared serum powder was then injected daily for seven days. The remaining

ovary was removed 24 hours after the last injection. In all animals the ovary removed first was almost completely free of precursor. However, unlike the finding of the preceding experiment in which $1/3$ IU Antex had been given, no storage had taken place in the remaining ovary. The interstitial gland showed a typical deficiency picture. *PMS by itself thus causes no precursor storage in the interstitial gland.*

Because of the slow elimination of PMS from the organism the experiment dealing with precursor storage on daily administration of $1/30$ γ PU is ambiguous. The small amounts of PMS which remain after the injection of 100 IU Antex can naturally have influenced the storage in combination with PU. This experiment, therefore, gives no information whether in this case — as to the oestrogen formation in the ovary (Fevold, 1939, 1941, 1943, 1944, Fraenkel-Conrat, Li, Simpson & Evans, 1940, Shedlovsky, Rothen, Greep, van Dyke & Chow, 1940, Greep, van Dyke & Chow, 1942) — it is a question of a synergism between two gonadotrophic factors, of the LH and the FSH character respectively. To obtain an answer to this question the experiment should be carried out by a method in which neither PMS nor any other FSH-factor remains in the organism when PU is given. Antex can, for instance, be substituted for PU to mobilise the precursor. The experiment was performed in the same way as the previous one. A control series of 10 animals was given 250 γ PU four days after hypophysectomy. One ovary was removed three days later as a control for the mobilization of precursor¹) and the other 8 days later to determine whether storage had occurred. The same mobilising dose was used in another series (5 animals), but here, after the removal of the first ovary, $1/30$ γ PU was injected daily for 7 days, and the second ovary was removed 24 hours after the last injection. — In all the animals of both series mobilization proved to be almost complete in the ovary

1) A close study of the biological purity of the electrophoretically homogenous chorionic gonadotrophin used and its capacity to mobilize the oestrogen-precursor will be published later. This problem will therefore not be discussed in the present paper.

removed at the first operation. The second ovary in both series, however, showed some precursor storage, but not in any way comparable with the marked storage occurring on the administration of $1/30 \gamma$ PU following an Antex mobilization. In the two experimental groups, however, there was no demonstrable difference in the precursor content of the interstitial gland. *Hence the administration of small amounts of highly purified PU to hypophysectomized rats does not produce a storage of precursor in the interstitial gland of ovaries in which the precursor has been previously mobilized by a large dose of PU.*

DISCUSSION

A chemical analysis of the oestrogen precursor has shown that it consists of cholesterol, stored in the interstitial gland in the form of esters of fatty acids (Claesson, Diczfalusy, Hillarp & Högborg, 1948). Whether this storage is an accumulation of blood cholesterol or whether an actual synthesis takes place in the cells cannot at present be determined. Bloch (1945), however, has demonstrated a direct conversion of deuterio-cholesterol into pregnandiol in a pregnant woman, this shows that the first alternative is possible. In any case the amount of precursor present at any given time must be determined by two mutually opposite processes namely, the process of storage and the conversion of the precursor into hormone. Hence, until the mechanism of storage and conversion is known, the amount of precursor present in a given case cannot provide a basis for an evaluation of the oestrogen formation in the interstitial gland.

Theoretically the precursor storage in the interstitial gland can take place in two different ways: either as an autonomous process in the cells independent of gonadotrophic hormones, or as a process which is directly or indirectly affected by these hormones. The present investigation seems to have excluded the first alternative, since no storage takes place in the ovaries of hypophysectomized animals after mobilization of the precursor by Antex. The experiments involving the administra-

tion of small amounts of Antex or of highly purified PU, however, indicate that gonadotrophic hormones have an effect on storage. These gonadotrophins produce a marked storage after previous mobilization of the precursor by a large dose of Antex.

It is doubtful, however, whether the PMS-preparation used does not also contain an LH-factor. It is true that *Hamburger* (1938) and *Cole, Pencharz & Goss* (1940) have found that highly purified PMS-preparations and crude preparations have the same biological effects, but *Evans, Korpi, Simpson & Pencharz* (1936), and *Hellbaum* (1937) have reported the separation of mare serum into follicle-stimulating and luteinizing fractions. The latter investigations also appear to be supported by the experiments with antigonadotrophic sera which were performed by *Kupperman, Meyer & McShan* (1941). The attempt to purify Antex biologically described in the present paper, also suggests that Antex contains some LH-factor. Otherwise it is difficult to explain why the purified PMS-product, when administered in the same dose as Antex, does not have the same effect on the precursor storage mechanism. In any case the experiment indicates that PMS by itself does not produce any such storage. This agrees very well with the fact that no storage occurs after an acute mobilization by a large dose of Antex. In experiments of this kind the PMS-concentration in the blood falls slowly while any LH-factor is presumably rapidly eliminated. PMS would thus be purified, i. e. freed from the LH-factor, automatically. If PMS had possessed any storage capacity, favorable conditions would exist for the process when the concentration falls below the mobilization level.

Neither does PU seem to produce a precursor storage by itself. This statement may appear to contradict the observation that a certain amount of storage takes place after an acute mobilization with a large dose of PU, but the amount used (250 γ), however, is so great that a possible contaminating FSH-factor may be involved. As our experiments are still proceeding it seems advisable to postpone the discussion of this problem to a later date.

The investigations so far performed indicate that only a combination of PMS and PU can produce a marked storage of precursor in the interstitial gland. This is evident from the experiments involving the administration of small amounts of Antex or of highly purified PU, after a previous mobilization with Antex (and from the fact that PMS and PU do not produce any such effect by themselves). In this respect a synergism exists between these two gonadotrophins.

Since no pure gonadotrophic hormones from the hypophysis have been available to us, we have not been able to investigate their role in the precursor storage mechanism. A study of this subject is of course, very necessary for the evaluation of changes in the precursor content of the ovary. From the present investigation the conclusion might tentatively be drawn that the precursor storage in the intact animal occurs as a result of a synergistic action of FSH and LH. This conclusion appears to be supported by the fact that PMS and PU have respectively the characteristics of FSH and LH. Such a conclusion is, however, somewhat premature, as these extrahypophyseal gonadotrophins possess certain biological properties which differentiate them from the gonadotrophic hormones of the hypophysis (cf., *Levin, 1944, Robson, 1947*).

SUMMARY

Investigations on the storage mechanism of the oestrogen-precursor in the ovary have been carried out on hypophysectomized adult rats. This mechanism must be known before the precursor content can be used as a basis for the evaluation of the oestrogen formation in the interstitial gland. — Pregnant mare serum gonadotrophin (not highly purified) (Antex Leo), and an electrophoretically homogeneous crystalline chorionic gonadotrophin (Gonadex Leo) have been used.

The investigations show that the precursor storage does not take place as an autonomous cellular process but is controlled by gonadotrophic hormone. Neither PMS nor PU alone seem to produce storage in the interstitial glandular cells, but

a combination of small amounts of PMS and PU produce marked storage of precursor. In this respect a synergism therefore exists between these two gonadotrophins.

A preliminary attempt to eliminate a contaminating LH factor from serum gonadotrophin by biological methods is described. Reasons are presented for the view that such a contaminating factor is present in Antex.

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CELL REACTIONS IN THE HYPOTHALAMUS FOLLOWING OVERLOADING OF THE ANTIDIURETIC FUNCTION*)

BY

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The secretion of antidiuretic hormone from the neurohypophysis is released in response to an increase in the osmotic pressure of the blood plasma (*Chambers, 1945, Chambers, Melville & Hare, 1945, Hare, Melville, Chambers & Hare, 1945, Hare, 1947, Plumhof, Stevenson & Toman, 1947*). Investigations, especially those of *Verney (1946, 1947)*, have demonstrated the presence of receptors within the area of the internal carotid which respond to such osmotic changes in the plasma. The exact location of these receptors is as yet unknown. *Ranson* and his co-workers (*Fischer, Ingram & Ranson, 1938*), however, have made it clear that the supraoptic nucleus is directly involved in the antidiuretic function of the neurohypophysis. Although it is probable that the paraventricular nucleus also innervates the neurohypophysis (*Frykman, 1942, O'Connor, 1947*), it has not been possible, however, to say whether it plays any part in the antidiuretic function of the posterior lobe of the hypophysis.

By means of intravenous, subcutaneous or oral admini-

*) Aided by a grant from the Faculty of Medicine, University of Lund.

stration of a hypertonic solution of sodium chloride to an animal the antidiuretic function can be heavily overloaded (Gilman & Goodman, 1937, Ingram, Ladd & Benbow, 1939, Chambers, 1945). This must result in increased activity in the hypothalamic nuclei of the neurohypophysis, if it is true that these cell groups constitute osmoreceptors, or are affected by such receptors. Increased activity in a nerve cell can as a rule be detected cytologically and it is thus possible to obtain more definite information as to which cell groups are involved in the secretion of the antidiuretic hormone. In the present communication the cytological changes found in the cells of the hypothalamus of rats, during acute or chronic overloading of the antidiuretic function, are described.

MATERIAL AND METHODS

The experiments were carried out on adult albino rats inbred for eight generations. The animals were divided into two groups (Table 1). The first group was injected intravenously (in the tail) or subcutaneously with 6—10 ml. of 5 per cent NaCl-solution. The animals were killed 10 minutes to 22 hours after the beginning of the injection, and during the interval they received no food or water. In the other group the rats were kept mainly on a dry diet consisting of mixed grain, offal, carrots, and water containing 1.5 per cent NaCl for the first week, and 2.0 per cent and 2.5 per cent for 2 weeks respectively. The control animals were given the same food but water without NaCl. All the animals were bled to death under light ether anesthesia. The brains were fixed in Carnoy's solution by means of injection into the carotid arteries. The hypothalamic region was embedded in paraffin, cut in serial sections of 10 μ , every fourth section being stained with gallocyanine-chromalum according to Einarson (1932, 1934, 1935, 1947). Since this staining method is progressive, it is far more satisfactory for the evaluation of the cytological changes in the nerve cells than the commonly used regressive staining obtained with cresyl violet, methylene blue, and other basic aniline dyes belonging to this group.

Table I.
Animal material and treatment.

No.	Sex	Weight gm.	Treatment	Killed after
I. Acute overloading.				
1	♂	210	6 ml 5 % NaCl intraven.	10 min.
2	»	220	6 » » » »	20 »
3	»	205	6 » » » »	30 »
4	»	200	6 » » » »	35 »
5	»	210	6 » » » »	1 hr.
6	»	225	8 » » » subcut.	2½ »
7	»	210	8 » » » »	3 »
8	»	215	8 » » » »	4 »
9	»	190	8 » » » »	5 »
10	»	180	8 » » » »	5 »
11	»	160	10 » » » »	6 »
12	»	195	8 » » » »	8½ »
13	»	165	8 » » » »	8½ »
14	»	200	8 » » » »	22 »
15	»	200	8 » » » »	22 »
II. Chronic overloading.				
18—19	♀	180—190	NaCl in the drinking-water	5 wks.
20—23	♂	210—240	» » » »	» »
24—25	♀	180—185	Controls	
26—29	♂	150—300	»	

RESULTS

I. Acute overloading.

The nerve cells in the supraoptic nucleus have a very characteristic appearance with aggregations of Nissl-substance in the periphery of the cytoplasm and little in the central portions (Fig. 1). No marked changes in this picture are seen until 2½ hours after the injection of NaCl-solution. By this time the Nissl-substance in the periphery has disappeared almost completely in a large number of cells. These changes were especially marked in animals killed 5 to 8½ hours after the injection. By then the cells all show great reduction in Nissl-substance, which is finely granular, evenly distributed, and faintly stained (Fig. 2). The picture is suggestive of the

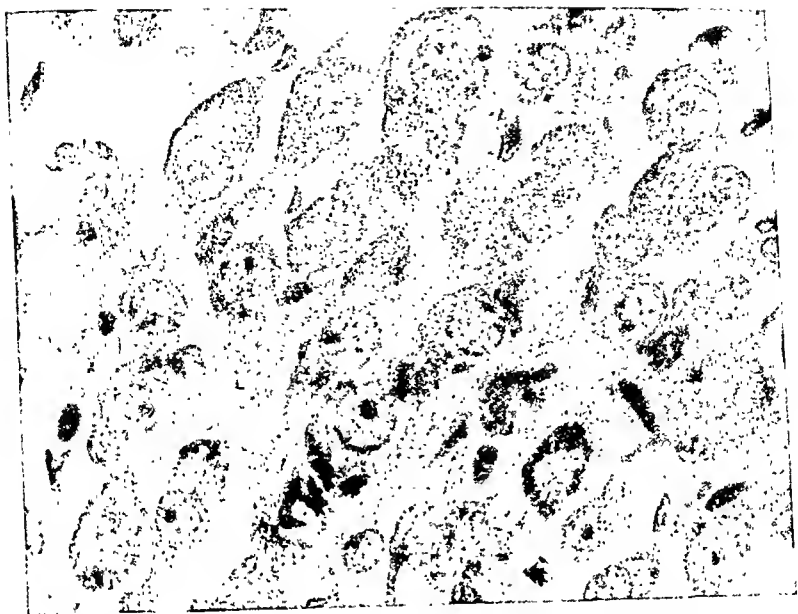


Fig. 1.

Normal cell picture in the supraoptic nucleus of rat. Control animal R 27. Gallocyanine-chromalum. 630 \times .

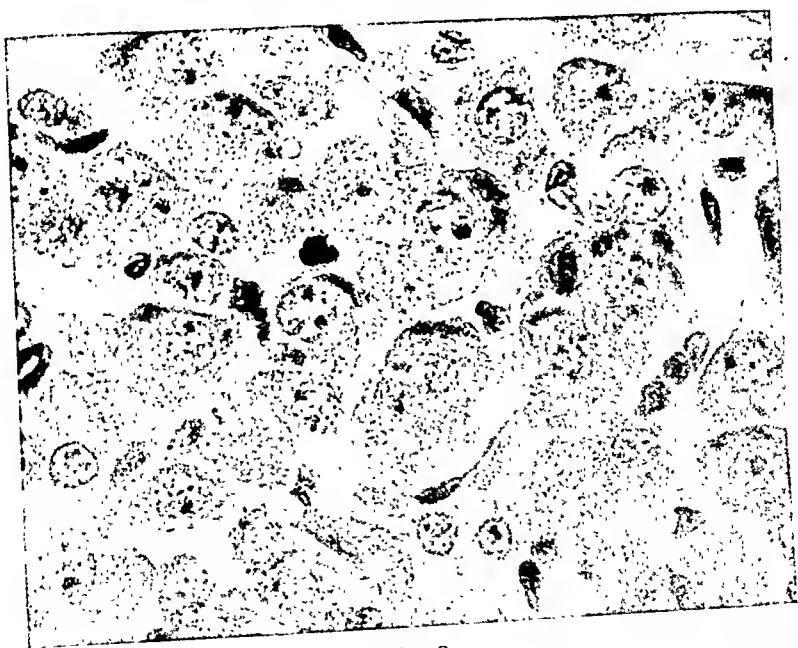


Fig. 2.

Nerve cells in the supraoptic nucleus of rat; 6 hours after subcutaneous injection of 5 per cent NaCl. The cells have undergone chromotolysis. Rat R 11. Gallocyanine chromatum. 630 \times .

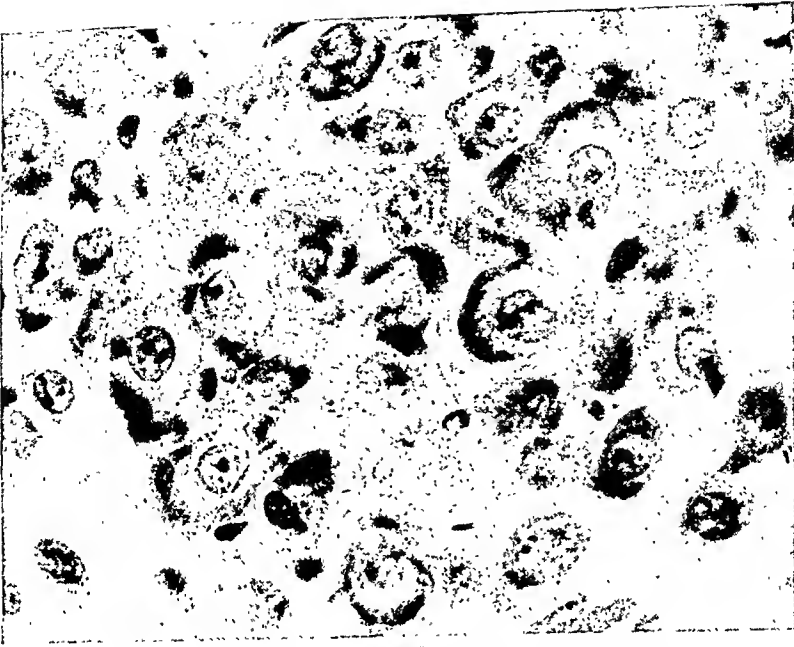


Fig. 3.

Normal cell picture in the paraventricular nucleus of rat. Control animal R 27. Gallocyanine-chromalum. 630 \times .

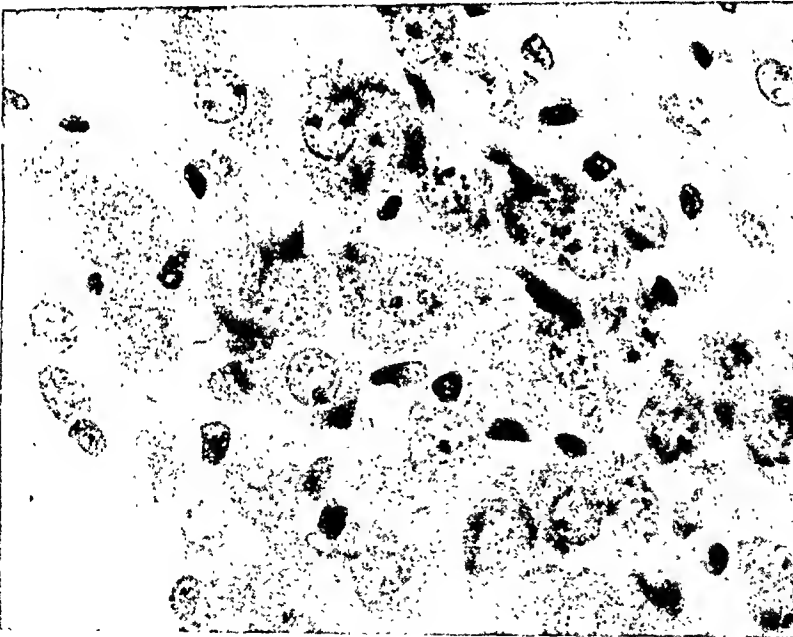


Fig. 4.

Nerve cells in the paraventricular nucleus of rat; 6 hours after subcutaneous injection of 5 per cent NaCl. The cells have undergone chromatolysis. Rat R 11. Gallocyanine-chromalum. 630 \times .

chromatolysis of the retrograde reaction. No definite changes were observed in the nucleoli and cell nuclei. After 22 hours, however, the cells have largely regained their normal appearance with abundant Nissl-substance in the periphery.

The nerve cells in the magnocellular portion of the paraventricular nucleus have normally the same distribution of Nissl granules as in the supraoptic nucleus (Fig. 3). Here, too, the cytoplasm passes through a definite, although not so marked, chromatolytic reaction, which is, however, not obvious until 6 hours after the injection of NaCl-solution (Fig. 4). After 22 hours the Nissl-substance is largely restored.

Thus, on acute overloading of the antidiuretic function of the posterior lobe of the pituitary gland a distinct chromatolytic reaction can be demonstrated in the nerve cells of the supraoptic nucleus as well as in the magnocellular portion of the paraventricular nucleus.

II. Chronic overloading.

Distinct changes in the cells of the supraoptic and of the paraventricular nuclei also occur as a result of an overloading which is slight when compared to the acute overloading described in I, but is instead much more prolonged. As in the previous experiment these two cell groups react in a similar way.

Although no actual measurements were made it is obvious from a comparison between the control and the experimental animals that the nerve cells have undergone definite hypertrophy (cf., Fig. 1 with Fig. 5 and 3 with 6). There seems to be an increase in the Nissl-substance consisting essentially of large aggregations, often coarsely granular, in the periphery of the cytoplasm. Finally, a close study of the size of the nucleoli shows that they have undergone considerable increase in volume. The procedure was as follows: The measurements were carried out by means of an echelon micrometer (oil immersion; approximately $1000\times$ magnification; each scale unit = $1/14\ \mu$). Only well defined nucleoli were measured. The

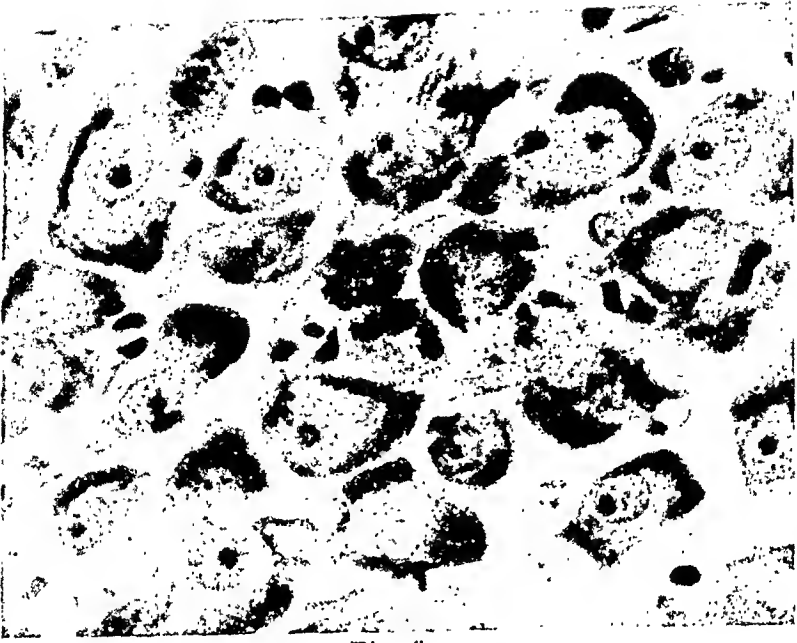


Fig. 5.

The cell picture in the supraoptic nucleus of rat following chronic overloading of the antidiuretic function. Hypertrophied cells with large nucleolus and abundant peripheral Nissl-substance. Rat R 21. Gallocyanine-chromalum. 630 X.

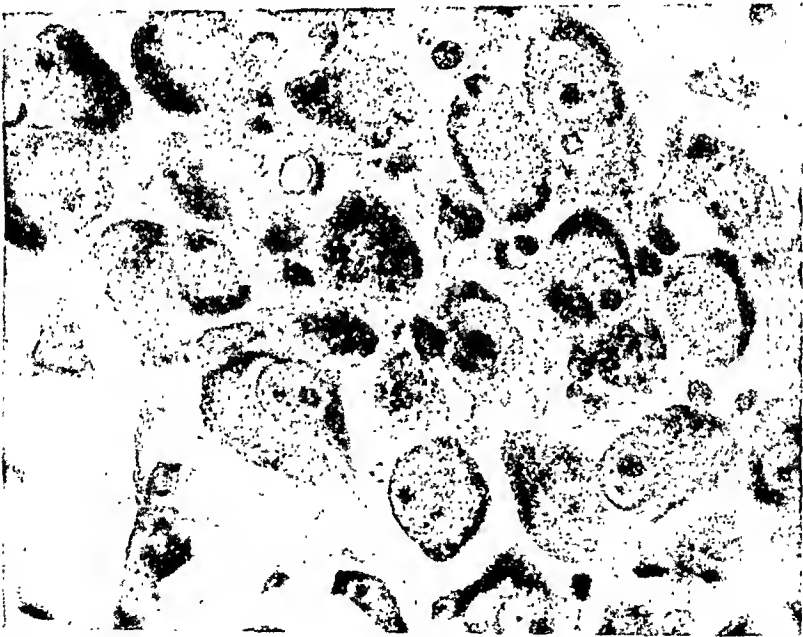


Fig. 6.

The cell picture in the paraventricular nucleus of rat following chronic overloading of the antidiuretic function. Hypertrophied cells with large nucleolus and abundant peripheral Nissl-substance. Rat R 20. Gallocyanine-chromalum. 630 X.

measurements were performed without selection, on all nucleoli which appeared within the field of vision on moving the preparations. In each animal a hundred nucleoli have been measured (50 in the right and left cell groups respectively) both in the supraoptic nucleus (within the middle portion) and in the paraventricular nucleus (within the dorso-lateral, magnocellular portion). The value obtained for the supraoptic nucleus in the control animals R 26 and R 27 was 46.5 ± 4.9 (expressed in scale units) and, in the experimental animals R 20 — 23, 60.5 ± 5.1 . There is thus a 30 per cent increase in the diameter. The values for the paraventricular nucleus were 43 ± 4.6 (R 24 — 27) and 53 ± 5.1 (R 18 — 23) respectively, that is to say, an increase in the diameter of 23 per cent.

During a relatively slight but prolonged overloading of the antidiuretic function of the posterior lobe of the pituitary gland marked cellular changes, especially in the form of cellular hypertrophy and an increase in the volume of the nucleoli, occur in both the supraoptic nucleus and the magnocellular portion of the paraventricular nucleus.

DISCUSSION

The supraoptic nucleus and the magnocellular portion of the paraventricular nucleus are in many respects similar in structure. Both are well-defined cellular areas with considerable vascularity (Finley, 1940), and have nerve cells of the same characteristic type. Furthermore they are in direct or indirect communication with the neurohypophysis. It has been known for a long time that the supraoptic nucleus innervates the posterior lobe of the pituitary gland directly, while the part played in this innervation by the paraventricular nucleus is still uncertain. It is true that some cell reduction occurs in this nucleus after stalk section, hypophysectomy or extirpation of the posterior lobe (Rasmussen, 1937, Magoun & Ranson, 1939, White & Heinbecker, 1939, Frykman, 1942, Heinbecker, White & Rolf, 1947, O'Connor, 1947), but it is not yet known whether this reduction is the result of a direct retro-

grade reaction, or of a transneuronal degeneration. A total disruption of the supraoptico-hypophysial fibre system causes diabetes insipidus (*Fischer, Ingram & Ranson, 1938*), and direct stimulation of the supraoptic nucleus — either electrically (*Harris, 1947*) or by acetylcholine (*Pickford, 1947*) — causes secretion of antidiuretic hormone from the neurohypophysis. Electrical stimulation of the paraventricular nucleus, however, does not have this effect (*Harris, 1947*). On the whole the function of this nucleus is obscure.

The present investigation indicates that both the supraoptic nucleus and the magnocellular portion of the paraventricular nucleus react in a similar manner to overloading of the antidiuretic function of the posterior lobe of the pituitary gland. On the basis of the known relation between the cytology and the state of activity of the nerve cells (cf., *Einarson, 1933, 1935, 1945, Einarson & Lorentzen, 1946, Hydén, 1943*) it may be inferred that the cellular changes in these nuclei following acute overloading represent a marked increase in activity verging on exhaustion and that the changes with chronic overloading represent an increased activity without any signs of exhaustion. It appears from several investigations, especially those of *Chambers (1945)*, that the method of overloading used produces a markedly increased secretion of antidiuretic hormone. It would, therefore, be reasonable to assume that the paraventricular nucleus too may be directly or indirectly connected with the antidiuretic function of the posterior lobe. Such a conclusion is, however, as yet premature. The experiments with acute overloading, in particular must be considered doubtful, since large amounts of 5 per cent NaCl solution are injected intravenously or subcutaneously. This procedure is bound to produce a number of compensating reactions in the organism, thus putting a strain on other functions besides the purely antidiuretic one. The chronic experiments, however, seem to be more satisfactory from this point of view, but even here the possibility cannot be disregarded that the cellular reactions in the paraventricular nucleus may be the result of overloading some function other than antidiuresis. Hence, the

only conclusion that can be drawn from the investigation is, that the cells in these two hypothalamic nuclei react in a similar manner, i. e. with increased activity, under the experimental conditions described, which cause an overloading of the antidiuretic and possibly other functions. — No cellular changes in other hypothalamic nuclei have been observed, but this does not exclude the possibility that they occur since the cytology of these nerve cells gives little indication of changes in their activity.

As far as the problem of the site of the osmoreceptors is concerned, the investigation suggests that they are not situated in the neurohypophysis itself. Otherwise the supraoptic nerve cells would in all probability not have been activated.

SUMMARY

Intravenous or subcutaneous injection of 5 per cent sodium chloride solution in rats, which causes a considerable acute overloading of the antidiuretic function of the posterior lobe of the pituitary gland, leads to a chromatolytic reaction in the nerve cells of both the supraoptic nucleus and the magnocellular portion of the paraventricular nucleus. Administration of NaCl in the drinking-water (1.5—2.5 per cent) for 5 weeks, which produces a relatively slight but prolonged overloading of the antidiuretic function, leads to changes in these nuclei consisting essentially of cellular hypertrophy and increase in the volume of the nucleoli. It is not certain, however, whether these reactions, which are an indication of increased cellular activity, on the part of the paraventricular nucleus, are the result of the overloading of the antidiuretic, or of some other function.

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THE EFFECT OF DESOXYCORTICOSTERONE
ACETATE AND SODIUM CHLORIDE, THYROXIN
AND TESTOSTERONE PROPIONATE IN A CASE
OF PANHYPOADENOPITUITARISM (SIMMONDS'
DISEASE) WITH SPECIAL REFERENCE TO
KIDNEY FUNCTION AND BLOOD
PRESSURE^{1, 2)}

BY

ROLF LUFT and BJÖRN SJÖGREN

INTRODUCTION

Simmonds' disease denotes a clinical syndrome, caused by a decrease in all the functions of the adenohypophysis. It has also been called panhypopituitarism. This name is not entirely satisfactory as it also includes a decreased activity of the neurohypophysis. We have preferred to introduce the term panhypoadenopituitarism in our case, as no histological examination could be made, and no signs of deranged function of the neurohypophysis could be found.

In animals the most important effects of hypophysectomy

1) The desoxycorticosterone acetate and testosterone propionate used in this work was Percorten and Perandren, and was kindly put at our disposal by Ciba Produkter A. B., Stockholm.

2) This case was kindly referred to us for treatment by Dr. Gustaf Myhrman, director of the medical department of the military hospital of Boden.

can be eliminated by the administration of pituitary extracts. In cases of panhypoadenopituitarism different types of pituitary preparations have been tried, mostly without therapeutic effect. In the Scandinavian literature *Mogensen* (1939, 1941) has claimed good therapeutic results with chorionic gonadotrophin in two cases of panhypoadenopituitarism. Doubtful or negative results with gonadotrophic hormones were reported by *Warburg* (1939), *Jersild* (1943), *Bierring & Iversen* (1943), and *Jersild & Iversen* (1944). *Lerman & Stebbins* in 1942, and *Darley et al.* (1944) reported good results with serum gonadotrophin in cases of panhypoadenopituitarism.

In panhypoadenopituitarism there is a secondary depression of the function of the peripheral endocrine glands. Many attempts have therefore been made to treat cases of panhypoadenopituitarism with the hormones of these glands.

The decreased thyroid function in panhypoadenopituitarism is shown by a very low BMR, and in some cases a pronounced myxedematous appearance is seen. Thyroid hormone has often caused toxic symptoms in this condition because of the increased sensitivity to this hormone. *Means et al.* (1940), therefore emphasize that thyroid hormone should not be used exclusively in these cases.

The deficiency of the gonads in these cases has been treated with oestrogenic hormones. However, this substitution therapy can not affect the main symptoms of the disease.

Adrenal cortical insufficiency is the most important disturbance found in panhypoadenopituitarism, and expresses itself in adynamia, hypotension, asthenia, negative protein balance, disordered water-salt balance, eosinophilia and lymphocytosis.

Cortical hormones, mainly total extracts or desoxycorticosterone acetate, have been tried in these cases. The improvement is usually slight and transient, but they have been effective in the acute stages of the disease (see *Means et al.*, 1940, *Glass*, 1944, and others). Thyroid hormone may aggravate the symptoms of adrenal insufficiency (*Means et al.*, 1940).

The androgenic hormones represent a valuable recent contribution to the treatment of panhypoadenopituitarism.

They possess the important capacity of increasing protein anabolism (*Kochakian & Murlin, 1936, Kenyon et al., 1938, Papanicolaou & Falk, 1938*). They also increase sodium and potassium retention (*Thorn & Engel, 1938, Thorn & Emerson, 1940*, and basal metabolism (*Kenyon et al., 1938, McCullagh, 1941*).

Good therapeutic results with androgens have been reported by a number of investigators, chiefly *Means et al. (1940)*, *Escamilla & Lissner (1942)*, *Williams & Whittenberger (1942)*, *Werner & West (1943)*, *Glass (1944)*, *Lissner & Curtis (1945)*, *Burke & Cantor (1945)*, and *McGavack et al. (1946)*.

In our case of panhypoadenopituitarism, we investigated the changes in blood pressure and kidney function after the administration of desoxycorticosterone acetate (DCA) and sodium chloride, testosterone propionate (TP) and thyroxin. Previously *Talbott et al. (1942)* and *McGavack et al. (1946)* had demonstrated a decreased kidney function in panhypoadenopituitarism in man, and the latter authors were able to restore the inulin clearance to normal with TP.

CASE REPORT

Past History.

M. C. G., a married woman, born 1909. Menarche at 15. Regular menstruations until 1938. Parturition 1938, complicated by a large post-partum bleeding, and a violent puerperal infection. After discharge from the hospital the patient gradually became lethargic, lost much weight and could not manage her regular house work. Her hair grew thinner, and she also lost pubic and axillary hair; the hair later grew again to some extent. There was no lactation after parturition, and menstruation never returned. The patient grew dull and apathetic, had great difficulties in finding words, and began to stutter. She was treated with vitamins and tonics in large doses. Her condition remained stationary until 1940. She was then, for the first time, treated in hospital, and was given large doses of oestrogenic hormones without any beneficial effect. Her condition deteriorated during the following years, the weakness, loss of weight and lack of initiative became more marked. At an examination in 1947 a marked hypothyroid condition was found with a BMR of -30 per cent, ichthyotic skin and marked apathy. With thyroid administration, the BMR rose to $+1$ per cent, whereas the body weight fell

from 52 to 37 kg. in one month. During this time she had a severe attack of hypoglycaemia with a blood sugar of less than 25 mg. per cent; her condition improved greatly following the intravenous administration of dextrose. There was also some physic improvement during this period, but thyroid medication had to be discontinued because of its toxic effects. Her tolerance to cold was very low. The internal genital organs were atrophic. X-ray of the skull showed nothing abnormal. ECG showed low T-waves with all leads. Urinary output was low. The haemoglobin was 50—60 per cent, and the blood pressure low. The hair loss was very marked during this period.

Physical Examination.

The patient was transferred to the endocrinological dept. in October 1947 (figs. 1—3). She was 158 cm. tall, and weighed 57 kg. She



Fig. 1.



Fig. 2.



Fig. 3.

Figs. 1—3. Case of panhypoadenopituitarism. The patient on admission to the hospital in November 1947.

was extremely thin with atrophic musculature. The face showed premature senility. The skin was dusky and pale. The mammary fat and glandular tissue was reduced. She was bald except for a sparse hair growth on the anterior part of the head. The remaining hair was dry, brittle and short. No signs of axillary and pubic hair growth. No abnormal pigmentations of skin or mucous membranes. Prothesis in the upper jaw, pronounced caries of the remaining teeth. Atrophy of the distal parts of the fingers, nails brittle. Thyroid normal. Superficial lymph glands not palpable. Normal findings in lungs, heart and abdomen. Blood pressure 105/65 mm. Hg. Neurological examination with normal findings. The patient was apathetic, lacking in initiative, answered questions slowly but adequately, and was well orientated. Gynaecological examination showed an atrophic uterus and vaginal mucous membrane, ovaries not palpable.

Laboratory findings. Hgbl. 64 per cent, red blood cells 3.2 mill., white cells 9.800. Diff. count: polymorphs 23 per cent, eosinophils 34

per cent, basophils 1 per cent, lymphocytes 40 per cent, mononuc. 2 per cent. Bone marrow normal with increased number of eosinophils. No albuminuria or glycosuria, spec. gr. 1.021. Faeces normal. Serum potassium 20—27 mg. per cent, sodium 280 mg. per cent, chlorides 332 mg. per cent, cholesterol 234 mg. per cent, calcium 9.9 mg. per cent, inorganic phosphorus 3.9 mg. per cent, phosphatase 6 Bodansky units. Albumins in serum 2.9 and globulins 3.2 mg. per cent. Serum iron 162 8/ml. NPN 44 mg. per cent. Haematocrit 38. Fasting blood sugar 90 mg. per cent. Calcium in urine 160 mg./day, phosphorus 1000 mg./day. Water test (1000 ml.) with dilution to a spec. gr. of 1.004 and concentration to 1.021, excretion in 4 hours 200 ml. Kepler's test index 20. BMR — 35 per cent. Inulin clearance 30 and diodrast clearance 147 ml./min. Dextrose tolerance (two-dose, one-hour test) 90 — 160 — 184 mg. per cent, capillary venous difference at 60 min. 8 mg. per cent. Insulin tolerance test (0.1 I. U./kg. body



Fig. 4.



Fig. 5.

weight intravenously) showed hypoglycaemic unresponsiveness. Oestrogen excretion less than 25 I. U./day, gonadotrophin less than 20 M. U./day, 17-ketosteroids 3.8 mg./day. Vaginal smears showed no cornification, and a large number of immature cells with nuclei.

Lumbar puncture: pressure 65 mm. water, chlorides 372 mg. per cent.

X-ray examination of the skull, skeleton, heart and abdomen gave normal findings. Old fibrous pulmonary changes.



Fig. 6.

Figs. 4—6. Same patient after treatment in September 1948.

Figs. 1—3 show the patient on admission to the hospital in November 1947, figs. 4—6 in September 1948.

A survey of the clinical and laboratory findings demonstrates the presence in this case of:

1. *gonadal hypofunction*: amenorrhea, genital atrophy, decreased oestrogen excretion and no oestrogenic stimulation of the vaginal mucosa;

2. *thyroid hypofunction*: very low BMR, skin changes as in hypothyroid conditions;

3. *adrenal cortical insufficiency*: hypotension, adynamia, weight loss, asthenia, loss of axillary and pubic hair, marked lymphocytosis and eosinophilia, hypoglycaemic unresponsiveness, decreased excretion of 17-ketosteroids, Kepler's test with an index of 20.

In spite of the ovarian insufficiency there was no increased gonadotrophin excretion, thus indicating a primary pituitary dysfunction. The decreased function of all endocrine glands examined, supports the suspicion of a primary panhypoadenopituitarism. This diagnosis is also supported by such facts as the age of the patient, the sex, the apathy, the premature senility, the time relation to a complicated delivery, and the absence of anorexia and gastro-intestinal disorders.

Treatment and Course.

A number of different treatments were given in successive periods, and this made it possible to determine the value of these treatments more accurately. A survey of these are given below and shown in fig. 7.

1. *Effect of 20 mg. DCA and 10 gm. NaCl daily* during 32 days. Blood pressure increased from 90/60 to an average of 110/70 mm. Hg. No significant change of haematocrit readings or number of red blood cells. Discontinuation of treatment was followed by a fall of blood pressure to 95/? mm. Hg. During this period the general condition improved, and she showed increased initiative, though still confined to bed. The adynamia remained.

2. *Testosterone propionate (TP) 25 mg./day* during 21 days, resulted in an increase in weight of 5 kg. and a further improvement of her general condition. The patient was able to get up for a few hours a day, and her appetite was much improved. There was no significant change in the BMR or serum cholesterol. No improvement of the axillary or pubic hair growth, nor of the hair growth on the head was seen. Blood pressure remained unchanged. Inulin clearance 30 and diodrast clearance 147 ml./min., corresponding to a renal blood flow of 226 ml./min.

3. *During the following 41 days the patient received 25 mg. TP and 3 tabl. of Thyroxin Roche à 1 mg. daily.* The body weight re-

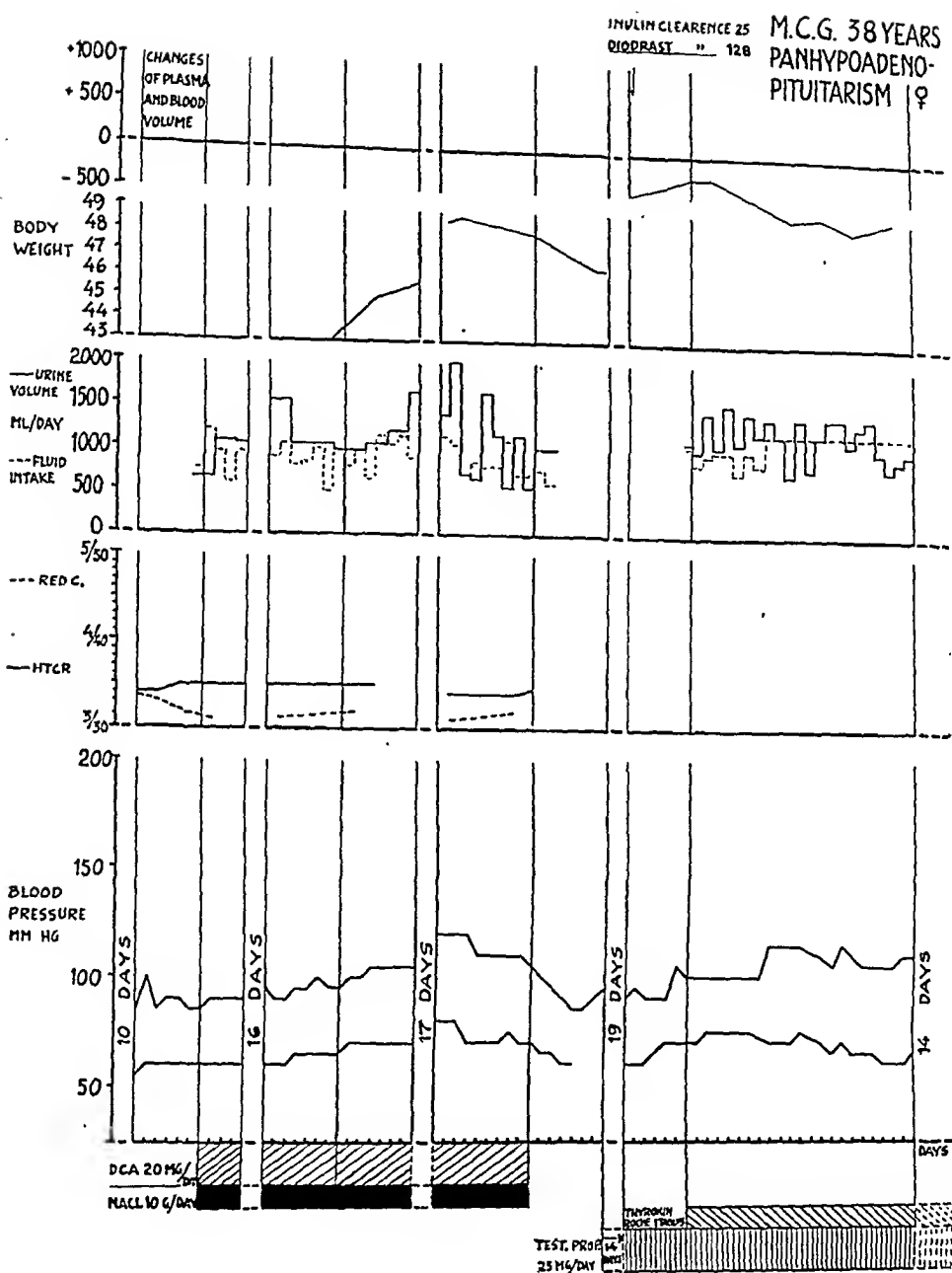
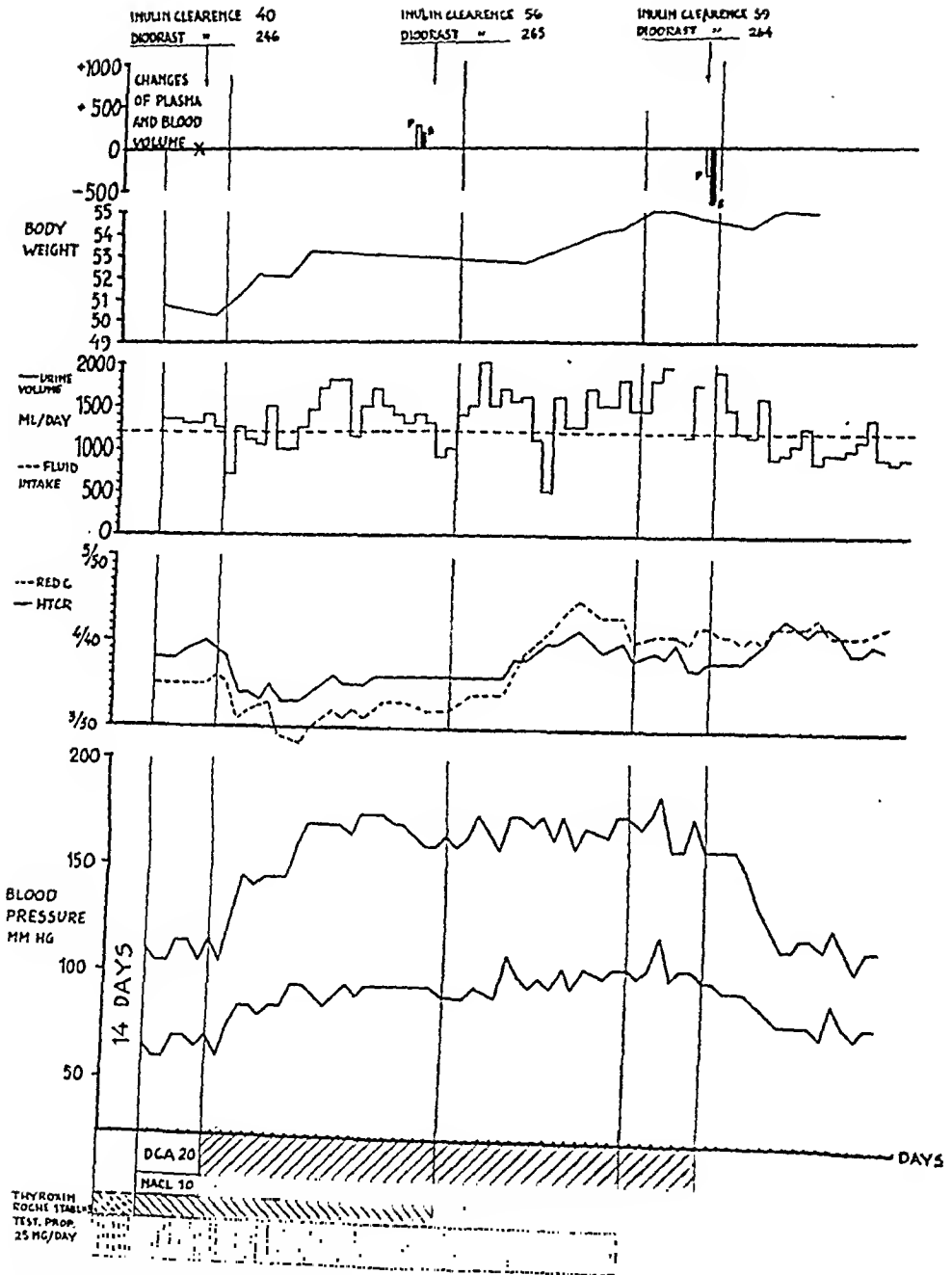


Fig. 7.

Effect of treatment on blood pressure, haematocrit readings and red



blood cell counts, fluid balance, body weight, volume of plasma and whole blood, and inulin and diodrast clearance.

mained at 50—51 kg. Her general condition was much improved, she showed increased activity. The patient was now up and about the whole day. The skin was rosy with signs of increased vascularity for the first time. Axillary and pubic hair reappeared at the end of this period. The BMR increased from — 45 per cent to + 8 per cent. Serum cholesterol decreased to 120 mg. per cent. Blood pressure remained at 110/65 mm. Hg. Inulin clearance 47, diodrast clearance 290 with a renal blood flow of 476 ml./min. The thyroxin treatment caused an increase in plasma volume of about 50 per cent.

4. *Thyroxin Roche 3 tabl., TP 25 mg., DCA 20 mg., and NaCl 10 gm. per day* for 22 days. Weight increased from 50.5 to 53 kg. There was further improvement of the general condition, the patient now being fully active. The BMR and serum cholesterol as in period 3. Rapid rise in blood pressure to 170/95 mm. Hg. Considerable fall of haematocrit readings and red blood cell counts, increase of these values towards the end of the period with unchanged blood pressure. Inulin clearance 64, diodrast clearance 304, renal blood flow 476 ml./min. Slight increase of the plasma and total blood volume as compared with the preceding period.

5. *TP 25 mg., DCA 20 mg., and NaCl 10 gm. per day* for 24 days. Cessation of thyroxin administration was followed by an increase in weight of 2 kg., marked decrease of the haematocrit readings and the red blood cell counts, while the blood pressure remained at 175/100 mm. Hg. The blood volume showed a marked decrease, and the fluid loss was greater than during any previous period. Inulin clearance 66, diodrast clearance 296, renal blood flow 469 ml./min.; serum cholesterol unchanged.

6. *Cessation of administration of TP, DCA and NaCl* caused a marked deterioration of the general condition. The patient was confined to bed for the greater part of the day. Blood pressure fell to 115/80 mm. Hg. Some rise of the haematocrit readings and red blood cell counts. BMR — 20 — 30 per cent, serum cholesterol 320 mg. per cent.

The patient was discharged, and 25 mg. TP and 20 mg. DCA intramuscularly were given twice a week, additional sodium chloride, thyroxin (Roche) 1 tabl. twice daily and vitamins. By this treatment she was able to keep up full activity. She returned four months later, when five tablets of Perandren and of Percorten (each 100 mg.) were implanted.

DISCUSSION

In this case the diagnosis of panhypoadenopituitarism was established beyond all doubt. There was a deficiency of the gonads, adrenal cortex, thyroid and glandular lobe of the pituitary, which had appeared following a complicated parturition. The symptoms progressed slowly.

The treatment resulted in an almost complete restoration of health. The patient could resume an active life and the lack of initiative was replaced by a normal psychic behaviour. It is of interest to note that these changes occurred after the patient had been an invalid for ten years. Similar therapeutic results have been obtained by earlier investigators in cases of panhypoadenopituitarism treated with androgens.

Certain observations made during the therapeutic experiments will be mentioned. The increased sensitivity to thyroid preparations, typical of the disease, was also observed here, showing itself by a rapid loss of weight, deterioration of the general condition, and a serious hypoglycaemic attack, even with a moderate dose of thyroid. This effect can be explained by the property of thyroxin of increasing the already high protein catabolism in these cases, and also of aggravating the symptoms of adrenal cortical insufficiency (*Byrom, 1934*). At a later period the patient was treated with thyroxin in combination with TP. Only on this occasion was the desired effect brought about i.e. the elimination of the hypothyroid component of the disease. This was probably due to the fact that TP was also given and this has a marked anabolic effect on protein metabolism, and also increases sodium retention (see above).

Prolonged administration of TP (25 mg. per day) did not produce any signs of hirsutism or virilism in this case. The administration of TP by itself for three weeks was not followed by any signs of axillary or pubic hair growth. When thyroxin was added, there was, however, a rapid development of pubic and axillary hair growth, and an improvement in the growth of the hair on the head. Simultaneously, the skin showed a markedly increased vascularity.

The blood pressure reactions were studied in detail. The patient showed hypotension, a symptom which always occurs in this disease. The diastolic pressure could not always be determined. Administration of 20 mg. DCA and 10 gm. sodium chloride per day was followed by only a slight rise in blood pressure. TP had no effect on the blood pressure, nor had TP in combination with thyroxin. However, when DCA and sodium chloride were given in the above doses, after TP and thyroxin had been administered for some time, a marked rise in the blood pressure (up to 170/100 mm. Hg) was seen.

With the administration of DCA, the changes in the fluid balance previously observed and described were obtained: an increased blood volume, decreased haematocrit readings and red blood cell counts, as well as water retention during the first days of medication. After cessation of thyroxin and TP administration, the blood pressure remained at the same high level despite the decreased blood volume, the increased haematocrit readings and red blood cell counts, and the increased fluid loss. It should be stressed, however, that the BMR remained high, and the cholesterol values showed only a slight increase, in spite of the discontinuation of thyroid for 24 days. A fall of the BMR was not observed until treatment with DCA ceased.

It has previously been shown that adrenal insufficiency in man is associated with impaired kidney function and low clearance values. During DCA treatment some improvement of the clearances have been reported (*Talbott et al.*, 1942, *McGavack et al.* 1946, *Waterhouse & Keutmann*, 1948). In our case we found no signs of chronic glomerulo-nephritis: there was no albuminuria, the urinary sediment was normal and the urine was concentrated to a spec. gravity of 1.021. Inulin clearance (Table 1) was 30 and diodrast clearance 147 ml./min. during the administration of TP, before the treatment of DCA, when the general condition of the patient was already greatly improved. Thyroxin increased the clearance values to 47 and 290 respectively, and the renal plasma flow remained at approximately this level throughout the experiment. This result

agrees with those reported by *Corcoran & Page* (1947), who investigated the effect of thyroxin on renal function in cases of myxoedema.

DCA increased the glomerular filtration to some extent but the renal blood flow remained constant (table 1).

Table 1.
Effect on Inulin and Diodrast Clearance during Different Periods of Treatment.

Period	Treatment	Inulin Clearance ¹⁾	Diodrast Clearance ¹⁾	Renal Blood Flow ¹⁾ ml/Min.	Filtration Fraction
2.	TP 25 mg./day	30	147	226	0.20
3.	TP 25 mg. + Thyroxin 3 mg./day	47	290	476	0.16
4.	TP 25 mg. + Thyroxin 3 mg. + DCA 20 mg. + NaCl 10 gm./day	64	304	476	0.21
5.	TP 25 mg. + DCA 20 mg. + NaCl 10 gm./day	66	296	469	0.22

1) The figures denote clearance values corrected for body surface (1.73 square meters of surface area).

Abbreviations: TP for testosterone propionate, DCA for desoxycorticosterone acetate.

In a recent paper the present authors have demonstrated that in certain cases, DCA in combination with sodium chloride can raise an initially normal blood pressure to that of hypertension (*Luft, Santesson & Sjögren*, 1948). In the same paper it was demonstrated that the hypertensive effect was not ne-

cessarily associated with an increase in blood volume, such as follows the administration of DCA. In this case too there was no relation between the changes in blood pressure and blood volume.

In the paper mentioned above the significance of an impaired renal function in the hypertensive effect of DCA was discussed. The possibility could not be excluded that the effect was, to a large extent, dependent on an impaired renal circulation. In the present case, DCA did not raise the blood pressure during the first period, when the renal function was very depressed. However, a significant hypertensive effect was produced by DCA at a later period, when the clearance values were greatly improved. This finding does not exclude the possibility that an impaired renal function plays an important part in the effect of DCA on the blood pressure. It seems that in the present case, the presence of a further factor was necessary for the production of the rise in blood pressure, and that this factor appeared after treatment with TP and thyroxin.

SUMMARY

The treatment of panhypoadenopituitarism (Simmonds' disease) is summarized. The therapeutic importance of androgenic hormones is emphasized. The main indication for their use is based on their property of increasing protein anabolism.

A case of panhypoadenopituitarism was treated with desoxycorticosterone acetate and sodium chloride, followed by testosterone propionate and thyroxin. The patient had been an invalid for the last ten years. After treatment she was almost completely restored to health — except for the ovarian function — and was able to carry on a normal life.

The effect of treatment is discussed, with particular reference to body weight, general condition, mental activity, BMR, condition of the skin, and hair growth. At first when the patient was treated with moderate doses of thyroxin alone, all the toxic symptoms characteristic of thyroxin were seen. Later when thyroxin was given together with testosterone propionate, no toxic effects occurred.

The effect of DCA and sodium chloride on the blood pressure was studied. When given alone, this treatment did not cause a rise in the blood pressure above the normal range. A marked hypertensive effect was, however, produced when DCA and sodium chloride were given after a period of administration of testosterone propionate and thyroxin.

Renal function measured with inulin and diodrast clearance was very poor before treatment and after administration of testosterone propionate. Thyroxin, however, almost doubled the glomerular filtration and renal blood flow; yet these values were still significantly lower than normal. DCA caused only minor alterations in the clearance values.

The significance of the alterations in blood volume and renal function in the hypertensive effect produced by DCA is also discussed.

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CAN THE ADMINISTRATION OF DESOXY- CORTICOSTERONE ACETATE GIVE RISE TO NEPHROSCLEROSIS?*)

BY

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Desoxycorticosterone acetate (DCA), the synthetic adrenocortical compound, is extensively used because of its ability to replace the adrenocortical hormones in adrenal insufficiencies of various kinds. The considerable rise of the blood pressure and the effect on the sodium-potassium balance of the organism caused by its administration in, for example, Addison's disease, encouraged attempts to induce an experimental rise of the blood pressure by means of large doses of the preparation. Many investigators have reported that a considerable rise of the blood pressure has been obtained both in experimental animals and in man with large doses of DCA given together with saline (*Engel et al.* 1942, *Ferrebee et al.* 1939, *Grollman et al.* 1940, *Perera et al.* 1944, *Roth et al.* 1943, *Soffer et al.* 1940, *Swingle et al.* 1941, *Thorn, Howard et al.* 1939, *Thorn, Dorrance & Day*, 1942). It is remarkable that

*) The present work was carried out with the aid of a grant from »P. Carl Petersens Fond«, Copenhagen.

both in animals and in man already suffering from hypertension a greater sensitivity to DCA than in normal subjects was found (*Kuhlmann et al.* 1939, *Perera & Blood*, 1947, *Rodbard & Freed*, 1942). Results do not, however, appear to be uniform and experiments have also been reported in which it was not possible to induce a rise of the blood pressure with DCA alone or combined with saline (*Braun-Menendez et al.* 1946, *Knowlton et al.* 1946, *Raab*, 1942). *Perera & Blood* (1947), with a daily dose of 10 mg. of DCA, obtained a rise of the blood pressure in subjects with hypertension, but succeeded in inducing it in normal individuals only after lengthy treatment.

Some workers consider that they have found changes in the adrenals in cases of essential hypertension in man (*Aubertin & Ambard*, 1904, *Schur & Wiesel*, 1907, *Philpot*, 1909, *Oppenheimer & Fishberg*, 1924, *Allen*, 1929, *Goldzieher*, 1931, *v. Locadou*, 1935, *Rinehart et al.* 1941). This fact, together with the hypertensive effect of large doses of DCA, has given rise to the assumption that the adrenals play a definite role in the development of this disease.

In order to throw further light on this question, *Selye* (1939—46) and his coworkers (*Dontigny et al.* 1946, *Hall & Selye*, 1945, *Hall et al.* 1946, *Masson & Beland*, 1943) have studied the effect of large doses of DCA in combination with saline on various experimental animals, chiefly rats. They found histological changes in the kidneys, involving the tubules, the interstitial tissue, the glomeruli, and the vessels. The effect of DCA and saline appeared to be increased very considerably if one kidney was removed before the start of the experiments, when the changes in the glomeruli and the vessels became particularly marked. The abovementioned investigators are of the opinion that these renal changes are similar to those found in subjects suffering from essential hypertension and that the administration of DCA can cause nephrosclerosis. They also consider that renal lesions of the same kind can be caused by physical strain of long duration or lengthy exposure to low temperatures. Under such conditions

there is also an enlargement of the adrenals, this is taken to indicate that the effect on the kidneys takes place through the adrenals. *Selye et al.* have summarized the results of their experiments as follows: A number of extrinsic factors (cold, strain, psychic irritation, etc.) cause a reaction in the body which presumably consists *inter alia* in an increase in the hormone production of the adrenal cortex (alarm reaction) which, on repeated and lengthy irritation, gives rise to changes in the vessels, particularly in the kidneys, similar to those found in essential hypertension.

Closer study of the illustrations of these investigators and their descriptions of the histological changes in the kidneys nevertheless raise doubts concerning their statement that a true nephrosclerosis has been produced. In mild cases the tubular lesions consist of a dilatation of the convoluted tubules with an increase in the size of the epithelial cells. Mitoses can at times be seen. There is also an increase in the weight of the kidneys. Sometimes protein casts are also seen in the tubules. The glomeruli may be somewhat enlarged but are usually unchanged. In more severe cases there is inflammatory oedema with many round cells around the renal pelvis, and in the interstitial connective tissue of the kidney. There is also atrophy of the tubular cells. Finally, in the most severe cases new connective tissue is formed in the stroma of the kidney; the glomeruli shrink and are hyalinized. Large scars may thus arise on the surface of the shrunken kidney.

The lesions are thus chiefly localized to the tubules and the interstitial tissue, whereas the glomeruli and the vessels only undergo changes in the most severe cases (very large doses of DCA). This is not in agreement with the conditions found in nephrosclerosis in which the lesions in the *arterioles* predominate even in the very early stages, whereas the lesions in the glomeruli and the interstitial tissue are not found until the disease (hypertension) has been present for some time. (In the experiments of *Selye et al.* the period of treatment did not as a rule exceed two months). Furthermore, it appears that renal lesions of the same type as those described by these

authors can be brought about in other ways. *Knowlton et al.* (1946) thus obtained only tubular lesions with DCA and saline, whereas they found lesions in the glomeruli and the interstitial tissue after the administration of serum containing antibodies against the renal substance given either alone or in combination with DCA.

Selye and his co-workers also describe damage to the cardiac muscles and the joints reminiscent of those found in acute rheumatic fever, as well as arterial lesions which they interpret as periarteritis nodosa caused by the administration of DCA in combination with saline, in unilaterally nephrectomised animals. Under the same conditions, cold and strain also produce similar changes.

Myocardial lesions of this kind have also been found in patients with Addison's disease treated with DCA, who died of cardiac insufficiency. They have also been demonstrated in experimental animals given a diet with a low potassium content. It therefore appears very probable that these lesions are caused by an insufficiency of potassium in the blood (*Currens & White*, 1944, *Darrow & Miller*, 1942, *Godolf & McBryde*, 1944) and that they are not identical with the myocardial lesions found in acute rheumatic fever. Periarteritis nodosa and acute rheumatic fever are generally considered to be allergic diseases in which the organic lesions are caused by an antigen-antibody reaction. The presence of similar vascular lesions in connexion with DCA treatment appears to us to indicate that the abovementioned workers have probably induced an allergy to DCA in their experimental animals, and that the lesions are thus not brought about by a specific effect of the substance administered.

We therefore consider it justifiable to discuss these questions briefly and to give an account of similar experiments made by us, particularly since a review of the work of *Selye et al.* has recently appeared in *Nordisk Medicin* (37, 89, 1948) in which the final conclusions of these investigators have, to large extent, been accepted.

EXPERIMENTS

1. (*Bergstrand*).

Ten male albino rats, weighing approximately 200 gm. were given daily subcutaneous injections of DCA in solution in oil («Doca», Pharmacia). The daily doses were 4, 2, 1, 0.5 and 0.25 mg. respectively. At the same time they also received 2—5 mg. subcutaneously, of physiological saline solution daily. Unilateral nephrectomy was not performed and they were given tap water to drink. The blood pressure was taken daily, using the method described by *Williams, Harrison & Grollman* (1939). One of the animals that was given 4 mg. of «Doca» daily, showed a slow rise of the systolic blood pressure from a mean of 120 mm. Hg before the experiment, to approximately 140 mm. Hg after 14 days. The blood pressure remained at this level until the end of the experiments (two months). Otherwise no changes in the blood pressure were observed in the experimental animals.

Histological examination of the kidneys revealed no changes in the glomeruli or in the vessels. There was no appreciable dilatation of the tubules. The epithelial cells of the convoluted tubules were usually large with much protoplasm. They contained large agglomerations of a structureless substance which stained deeply with acids. Here and there lysis of the nuclei and disintegration of the epithelial cells were seen. No casts of any kind were observed in the lumen of the tubules. The lesions corresponded in appearance to the degenerative phenomena with hyaline granules occasionally observed in the tubular cells. They could also be seen, although to a considerably lesser extent, in untreated control animals. There were no inflammatory changes in the interstitial connective tissue.

2. (*Bechgaard*).

Unilateral nephrectomy was performed on 15 albino rats, weighing approximately 200 gm. One pellet containing 20 mg. of DCA («Percorten», Ciba) was implanted subcutaneously in 10 of the animals. Five rats were kept as controls. All the ani-

mals were given 0.9 per cent saline solution to drink during the entire period of the experiment (4 months). One animal in the experimental group and one of the controls died shortly after the start of the experiments. Using the same method as in the first experimental series, recordings of the blood pressure showed a mean rise of 18 mm. Hg in the systolic pressure. Microscopic examination (performed by *H. Gormsen*) of the kidneys revealed dilatation of the tubules without epithelial damage in four of the experimental animals and in one control. No damage to the glomeruli or to the vascular system could be seen. In one case, however, small infiltrations of lymphocytes were observed surrounding a few slightly shrunken ischaemic glomeruli.

During the course of the experiments the mean increase in body weight was 50 gm. for the controls and 26 gm. for the experimental animals. Finally the post mortem examination revealed insignificant remains of unabsorbed pellets.

DISCUSSION

It is thus seen that we were unable to induce any definite or constant rise of the systolic blood pressure by means of the administration of DCA together with saline. In those cases in which unilateral nephrectomy was performed there was a slight rise in the systolic pressure. In the kidneys, a dilatation of the tubules was found in a few cases and in others, degenerative changes in the epithelium of the convoluted tubules could be observed. On the other hand, we were unable to demonstrate any damage to the glomeruli or to the vascular system.

We used somewhat smaller quantities of DCA than those usually administered by *Selye et al.* These writers have, nevertheless, described renal lesions, which they interpreted as nephrosclerosis, even with doses as small as those used in our experiments. *Swingle et al.* (1941) also state that the maximum effect of DCA on dogs was produced with doses not exceeding 0.5 mg. per day.

Our experiments were only performed on a small scale and

not under the best conditions. Nevertheless, we are of the opinion that, without further investigation of this problem, the statement that »large doses of DCA (possibly with the simultaneous administration of saline and unilateral nephrectomy) can give rise to nephrosclerosis« must be regarded with scepticism.

By this statement we do not mean that DCA has no effect whatever on the kidneys. In agreement with other workers we have found changes in the renal tubules in some of our experimental animals. Nevertheless we consider it doubtful whether these changes can be entirely attributed to a specific effect of DCA. Similar change have been found after the administration not only of other hormones of similar chemical structure but also of substances of completely different constitution (*Longcope, 1913*).

SUMMARY

Ten albino rats were treated daily for two months with subcutaneous injections of 4.0—0.25 mg. of desoxycorticosterone acetate in oil (*Pharmacia*) together with 2—5 ml. of saline. No definite rise of the blood pressure was observed after two months. Slight degeneration of the tubular cells of the kidney was found in some cases. No changes in the renal vascular system were observed.

Ten unilaterally nephrectomized albino rats were treated with subcutaneous implantation of pellets containing 20 mg. of desoxycorticosterone acetate (*Ciba*). They were given saline instead of drinking water. In the course of four months, only a slight rise of the blood pressure occurred.

Slight dilatation of the tubules or degenerative changes in the tubular epithelium was observed in some cases, but there were no changes in the vascular system or in the glomeruli.

It is concluded that under these conditions desoxycorticosterone acetate may have a slight effect on the blood pressure, but that this is neither constant nor marked. Furthermore, the damage caused to the kidney is not of the same type as that found in malignant hypertension (nephrosclerosis) in man.

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THE CORTICOSTEROIDS, WITH SPECIAL
REFERENCE TO THE URINARY EXCRETION
IN NORMAL AND PATHOLOGICAL CASES
A SURVEY

BY

MOGENS SPRECHLER

During the last two decades interest has been increasingly focussed on the adrenal cortex and its special hormone production, under normal as well as under pathological conditions.

As far back as 1855 *Addison* described the typical clinical picture of adrenal cortical insufficiency, and localized the site of the disease. One year later *Brown Sequard* carried out the first experimental adrenalectomies; he found that the animals died, but did not discover the real cause of this effect.

It was not until 1913 that *Biedl* established that the adrenal cortex (AC), and not the medulla, is essential for the maintenance of life, and that hormones other than adrenaline are secreted in the adrenals. As experimental animals he used a species of fish in which the two components of the adrenal are separated.

Addison's disease was first treated with adrenal cortical extract by *Osler* in 1896, but attempts to produce sufficiently pure extracts in quantities to be of use clinically were only successful many years later.

In 1925 *Rogoff & Stewart* showed that adrenalectomized dogs and cats could be kept alive for a long time by the administration of AC-extract. This finding was confirmed by *Hartman et al.* (1928) and by *Swingle & Pfiffner* (1931).

In subsequent years *Grollman & Firor* (1933), *Kendall et al.* (1935) and *Wintersteiner & Pfiffner* (1935) were successful in isolating several crystalline compounds from the AC of cattle. The substances obtained were mixtures, but were sufficiently pure to be identified as steroid hormones. The greatest difficulty encountered in the production of the extract was the removal from it of various toxic impurities, especially adrenaline without diminishing its activity. In 1936, however, *Cartland & Kuizenga* described a method for the large scale preparation of AC-extract by the extraction of beef adrenals with acetone. After a complicated purification process they ultimately produced an extract, each ml. of which represented 40 gm. of adrenal gland and contained 0.6—1.0 mg. of solids. Dissolved in 0.9 per cent NaCl and 10 per cent ethanol and kept at a temperature of 4° C. it remained stable for at least a year. *Kuizenga* (1943) found that extracts prepared from pig adrenals possessed much higher biological activity.

The pure crystalline AC-hormones have been isolated from such extracts. Thus *Kendall and co-workers* isolated dehydrocorticosterone and corticosterone in 1936. The latter substance was almost simultaneously isolated by *Wintersteiner & Pfiffner* and by *Reichstein*. These investigators later isolated most of the 28 different adrenal cortical steroids now known. *Reichstein* has been particularly successful in this work, using Girard's reagents and subsequent chromatographic analysis.

THE CHEMICAL STRUCTURE

According to *Reichstein & Shoppee* (1943) all AC-steroids hitherto isolated can be roughly divided into three groups according to the number of carbon atoms, and it is found that 24 belong to the C_{21} group, 3 to the C_{19} group and one to the C_{18} group. The C_{21} group may be further subdivided by the

number of oxygen atoms present; the following groups have been found: 8 ($C_{21}O_5$), 9 ($C_{21}O_4$), 5 ($C_{21}O_3$) and 2 ($C_{21}O_2$). The adrenal cortical steroids all contain the fundamental nuclear skeleton, i. e. the perhydrocyclopentenophenanthrene ring system in common with the sterols, bile acids and sex hormones. In the present paper only the six steroids from AC which have a hormonal activity specific for this gland and so far identified will be mentioned in detail. They are all derived from pregnane, which is a 17-ethyl derivative of ethiocholane.

A few features of the structure will be more fully discussed as they are often of great significance in the biological activity of the compound.

The methyl groups attached at C_{13} and C_{10} are always on the same side (cis relation) and above the ring system, which is considered to be in the plane of the paper. The bond from the carbon atom to the substituent is then shown as a solid line. It is customary to place the configuration of all other substituents in relation to the given methyl groups, so that those lying on the same side as these are said to have the (β) configuration, shown as a solid line, while those lying on the opposite side of the molecule have the (α) configuration, and are shown by a short broken line for the bond connecting the substituent to the carbon atom. Ring A (Fig. 1) is unsaturated between C_4 and C_5 , this is shown by two solid lines and frequently referred to in formulae by Δ^4 ; the superscript refers to the carbon atom from which the double bond originates. C_{18} and C_{19} are applied to the carbon atoms in the methyl groups at C_{13} and C_{10} . The carbon atoms of the ethyl group attached at C_{17} has been called C_{20} and C_{21} .

Shoppee (1947), *von Euw & Reichstein* (1947), and *Mason*, (1948), have established the position of the various substituents in the molecule, so that we now know that the side chain of C_{17} always has the (β) configuration. This also applies to the hydroxyl group at C_{11} , if such be present, while the hydroxyl group at C_{17} in the three compounds mentioned has the (α) configuration.

The hormonal activity of AC steroids is apparently greatly

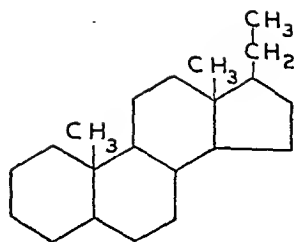
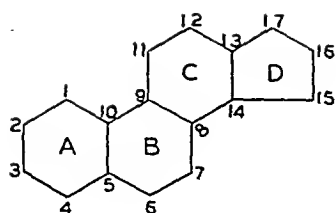
dependent on certain features of the molecular structure, of which the following may be emphasized:

- 1) the 3-keto- Δ^4 -group (called a α , β -unsaturated ketone group),
- 2) the 17-(β)-orientated side chain $-\text{CO}.\text{CH}_2\text{OH}$,
- 3) the hydroxyl or ketone group attached at C_{11} .

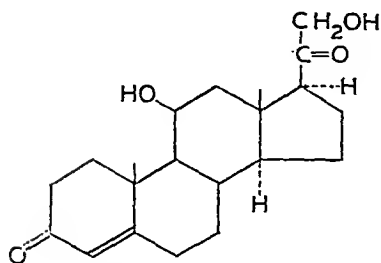
The two former groups are present in all the physiologically active AC steroids. A few compounds also contain 3), which seems to be essential for the effect of the steroid on carbohydrate and protein metabolism, and on the muscular activity.

Fig. 1 shows the structural formulae of active corticosteroids so far isolated from AC extract. They have, however, only a small fraction of the total activity found in the gland concentrate. After crystallisation of these steroids, the so-called amorphous fraction remains, which according to various investigations, contains up to 80 to 90 per cent of the total biological activity of the concentrate. In addition to 11-dehydrocorticosterone *Lowenstein & Zwemer* (1946) have further isolated two incompletely defined substances from concentrates of AC extract. The total biological activity of these substances represent 80 per cent of the original extract. One of the substances is claimed to be a ketonic steroid having the empirical formula $\text{C}_{25}\text{H}_{34-36}\text{O}_9$, and yielding ascorbic acid on mild anaerobic analysis. The substance had no specific biological effects, suggesting that it was not pure. Finally, *Hartman et al.* (1947) claim to have isolated, by chromatographic adsorption, an AC steroid which has an effect on the deposition of fat in the liver during inanition. This substance has, however, not been definitely characterized.

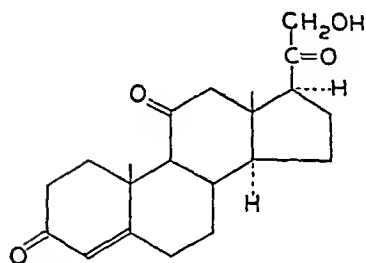
In addition to the above mentioned steroids, several others have been isolated which are biologically inactive and may represent conversion products or precursors of the biologically active steroids; finally, it should not be forgotten that AC in addition produces adrenosterone which has an androgenic effect, and also oestrone and progesterone.



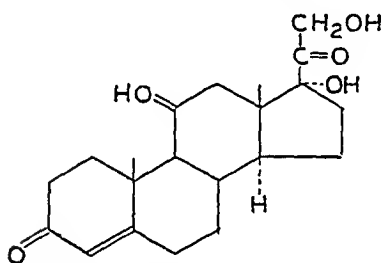
PREGNANE



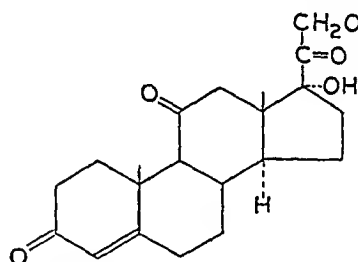
CORTICOSTERONE



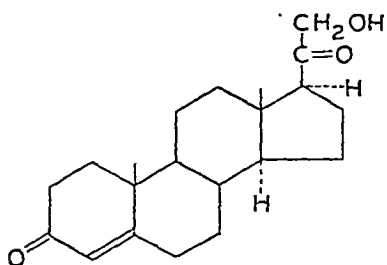
11-DEHYDROCORTICOSTERONE



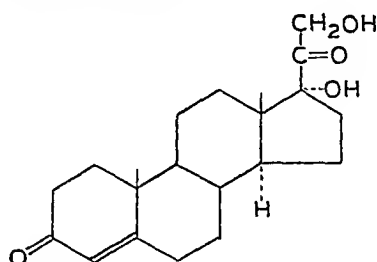
17-HYDROXYCORTICOSTERONE



11-DEHYDRO-17-HYDROXYCORTICOSTERONE



DESOXYCORTICOSTERONE



17-HYDROXY-DESOXYCORTICOSTERONE

Fig. 1.

The structural formulae of biologically active corticosteroids so far isolated from AC extract.

The production of the extract and the subsequent isolation of the individual substances have shown that the hormone content of AC is very small. Thus *Kendall* (1942) made quantitative determinations of 10 different steroids in an extract produced from 500 kg. of beef adrenals, and found amounts of biologically active substances ranging from 100 to 300 mg.; the amounts of desoxycorticosterone (DOC) were however negligible.

The preparation by *Steiger & Reichstein* in 1937 of DOC in vitro from the plant sterol, stigmasterol, was therefore an important advance. Later a series of the previously isolated AC steroids were synthesised including the active C_{11} -oxygenated substances with the exception of 17-hydroxycorticosterone. The syntheses have been made essentially from desoxycholine acid and cholesterol. They are all partial syntheses, since it has not yet been possible to synthesise the steroid nucleus. The basic material, therefore, must always be the natural substances possessing the typical »4-ring system«.

THE ADRENAL CORTICAL HORMONE

The finding of so many steroids with such different activities in the adrenal cortex has given rise to a good deal of discussion as to their true nature, with particular reference to the question: 1) whether there is one substance which is the adrenal cortical hormone proper, or 2) whether AC forms qualitatively different hormones, as in the case for the pituitary gland. The histological structure of AC is not uniform and this is in keeping with the latter view. Most investigators are in favour of a dualistic view, partly because of the great difference between the effect of DOC and the C_{11} -oxygenated corticosteroids, and also because of the many different deficiency symptoms which follows adrenalectomy, and which cannot be ascribed to the absence of any one of the known steroids. A few authors have claimed that there are other factors, e. g. *Hartman's* sodium factor and permeability factor,

but, like *Kendall's* life-maintenance factor, these can all be fitted into the dualistic theory.

Verzar, in particular, advocates the unitarian theory stressing that DOC possesses all the properties of cortin. Against this certain objections have been raised, 1) that DOC has only a slight effect, or none at all, on carbohydrate metabolism, 2) that occasionally patients with Addison's disease fail to respond completely to DOC alone, when in combination with AC-extract it is effective, and 3) that there is some doubt, whether DOC is actually present in the body. The whole question has been further complicated by recent studies which show that the secretion of hormones in the adrenal cortex is much greater than the amounts which can be isolated from the glands. This suggests that the hormone is not stored in the AC, but is being continuously synthesised from inactive precursors and immediately poured into the blood stream. This is particularly shown by the investigations of *Vogt* (1943, 1944, 1947), who determined the hormone content in the venous blood from the adrenals of dogs and, in the course of these studies, found that the 24-hour output of both adrenals in a dog weighing 10 kg. corresponded to the hormone content of 17 kg. of glands. *Vogt* calculated that this amount corresponded to about 54 mg. of 11-dehydro-17-hydroxycorticosterone or 1000 times the total daily excretion of biologically active substances in the human urine. Similar values have been found by others, e. g. by *Olson et al.* (1944). At the same time it was established that cortin quickly passes from the blood stream into the tissues, even in nephrectomized and eviscerated animals. Furthermore, it was shown that there was an increased excretion of hormones from the adrenals following the injection into the animal of amounts of adrenaline similar to those levels following splanchnic stimulation (6—8 μ g. per kg.). The increased excretion occurred rapidly and was of very short duration.

It would be outside the scope of this article to deal in greater detail with the mode of action of the adrenal cortical

hormones, especially as so much about this subject is still obscure. On the whole, it has been possible to attribute the changes which follow adrenalectomy to disturbances in the carbohydrate metabolism (*Boggild*, 1925; *Evans*, 1941; *Abelin*, 1946; *Jonas*, 1946), protein metabolism (*Long*, 1942) and electrolyte metabolism (*Heni*, 1947). This finding, together with the results of experiments with the individual pure crystalline steroids make it possible to group the biologically active AC steroids as follows:

- I) Corticosteroids with no oxygen attached at C₁₁, having a specific effect on water and electrolyte metabolism. These steroids may be called mineralo-corticoids. Desoxycorticosterone and perhaps the amorphous fraction belong to this group.
- II) Corticosteroids with oxygen attached at C₁₁, having a specific effect on protein and carbohydrate metabolism. They have been named: Glucocorticoids. Corticosterone, 11-dehydrocorticosterone and their 17-hydroxy derivatives belong to this group.
- III) Steroids having sex hormone actions, including the androgens, the oestrogens and progesterone.

This division into groups according to the effects produced is by no means absolute, but only express the most important property of any given substance. Thus glucocorticoids also have some effect on water and salt metabolism.

BIOLOGICAL TESTS FOR THE DETERMINATION OF THE CORTICOSTEROIDS

These may be listed as follows:

- I) a) The life-maintenance test
 - in the dog: *Swingle & Pfiffner*, 1932.
 - in the rat: *Kutz*, 1931.
 - Grollman & Firor*, 1933.

b) The life-maintenance and growth test
Cartland & Kuizenga, 1936.
Kuizenga, 1943.

c) The growth test
Grollman, 1941.

d) The cold test
Selye & Schenker, 1938.

The cold test modified
Tyslowitz & Astwood, 1942.
Ross, 1943.

II) Carbohydrate tests (which measure the glucocorticoids):

a) The liver glycogen deposition tests
Reinecke & Kendall, 1942.
Olson et al., 1944.
Eggleston et al., 1946.
Dorfman et al., 1946 a.
Venning et al., 1946.

b) The diabetogenic test on intact rats
Ingle, 1941.

c) The diabetogenic test on partially pancreatectomized rats
Ingle, 1941.

d) The anti-insulin test
Grattan & Jensen, 1940.

III) The muscular performance tests:

a) The muscular work test of
Everse & de Fremery, 1932.

b) The muscular work test of
Ingle, 1938, 1944.

c) The swimming test
Gaarenstroom et al., 1937.

IV) The test of the renal function in adrenalectomized dog
Pfiffner, Swingle & Vars, 1934.

The test of sodium retention in normal dog
Hartman et al., 1941.

The above test modified by use of radiosodium
Murphy & Dorfman, 1947.

The potassium excretion test in normal animals
West, 1942.

The test of semicontraction of melanophores of the carp's
 scales

Giroud, Santa & Martinet, 1939.

Several tests which measure the increased resistance to
 intoxications, poisoning and shock

Eversole, Gaunt & Kendall, 1942.

Perla & Gottesman, 1931.

Feil & Dorfman, 1945.

Lewis & Page, 1946.

Elmadjian & Pincus, 1944.

Of all the tests mentioned above, only the carbohydrate tests are according to the present findings specific in the determination of a particular group of corticosteroids, viz. the glucocorticoids, which constitute perhaps the most important fraction of cortin. These steroids are also the most potent in the muscular performance tests, but as some authors have also found a DOC effect in experiments with these tests, it will be necessary to retain them in a special group. In the tests (I) and (IV) the activity of all the known biologically active corticosteroids are shown. The tests most commonly used have been: I d, II a, III a and b.

The cold test is based on the great sensitivity to cold of adrenalectomized rats, and the ability of the AC hormones to increase the resistance of these animals under such conditions, the prolonged survival observed after injection of the hormones being used as a criterion of the effect. It is claimed

to be the most sensitive of the tests, allowing of the determination of as little as 10 μ g. of corticosterone. Most investigators find, however, that animals vary greatly in sensitivity from day to day, and the individual results of each experiment show a great dispersion; a large number of animals and much extract are required, and it is advisable to use a standard dose in each assay. *Dorfman et al.* (1946 b) were able to demonstrate the effect of 0.05 ml. of AC extract. Extract from the urine of male subjects were found to be active in doses corresponding to 15 to 40 ml. of urine, but in the case of larger doses there was a steep decline in the log dose-response curve, suggesting that a toxic factor was involved which further restricts the practical application of this method. The present studies show that this method would be even less satisfactory for the routine determinations of the urinary excretion of corticosteroids. In the cold test all the corticosteroids which have a life maintaining effect on adrenalectomized animals are assayed.

The liver glycogen deposition tests are in principle based on the inability of the fasting adrenalectomized animal to form carbohydrate from protein, while at the same time the oxydation of carbohydrate in the peripheral tissue is accelerated, resulting in a depletion of the glycogen depots, especially those of the liver.

As far back as 1932 *Britton & Silvette* showed that AC extract was able to restore the blood sugar and the liver and muscle glycogen depots. But it was only in 1942 that *Reinecke & Kendall* developed a method for the quantitative determination of the AC hormone, which was based on this effect. *Olson et al.* modified this method in 1944. These investigators showed that the hormone is best given in saline or in 10 per cent alcohol, and that the injection in an oily medium gave only a 50 per cent utilisation of the hormone or extract. Rats were used in these methods of standardisation, which were, however, partly abandoned, because their sensitivity was not sufficiently great for the determination of the small amounts of active substances normally present in urine. *Long, Katzin &*

Fry (1940) observed that the injection of AC extract into mice brought about a very high deposition of glycogen in the liver, and on the basis of this finding, two methods were developed in 1946, one by *Venning, Kazmin & Bell*, the other by *Eggleston, Johnston & Dobriner*. In both methods adrenalectomized mice are used, the experiment starting on the 4th postoperative day. In the former method the livers of the mice are depleted of glycogen by a preceding fast; in order to obtain a greater sensitivity, glucose is administered together with the hormone. The dose of glucose should be such as not to cause any deposition of glycogen in the liver when administered alone. The total dose of the extract is given in 7 injections spread over 5½ hours. One hour later the liver is removed and the glycogen content determined. The results are expressed in mg. of glycogen per 100 gm. of body weight. The assay is performed using 11-dehydro-17-hydroxycorticosterone as a standard. The biological activity of 1 µg. of this substance = 1 glycogenic unit. In the latter method the experiment is started with a certain level of glycogen in the liver, which is achieved either by injecting a certain amount of AC extract on the third postoperative day or by not removing the food until immediately before the first injection of the experiment. In this method the ability of the hormone to maintain the glycogen level is determined, while in the former method the ability of the hormone to produce deposits of glycogen in the liver is determined.

Both methods are considerably more sensitive (at least 50 times) than the original method described by *Reinecke & Kendall*, and it is thus possible to determine the small amounts of active substances present in urinary extracts. In the experiment there is some individual variation to the response, which is, however, not so great as to prevent fairly uniform results being obtained by the use of 6 to 10 animals in each experiment. *Thayer* (1946), using rats, found an error of ± 20 per cent in the assay, and stated this is somewhat higher in experiments with mice.

A number of findings have been published on the relative

activity of the various crystalline corticosteroids, e. g. by *Olson et al.* (1944), *Pabst et al.* (1947) and *Dorfman et al.* (1946 a). Most investigators find only a slight difference between the activity of corticosterone and its 11-dehydro derivative, while 11-dehydro-17-hydroxycorticosterone is two or three times so potent. The log dose-response curves of these substances are parallel, and it is interesting to note that the curve for urinary extracts has a similar slope, so that in fact they can all be used as standards. The 17-hydroxycorticosterone is the most active, but it differs somewhat from the others, and this is seen clearly by comparing the log dose-response curves, which is very steep for the latter substance. It has been suggested that the oxygenation at C₁₁ is of special significance in this connection. It should be mentioned that 17-hydroxycorticosterone was also found most active in the work test (*Ingle*, 1944; *Ingle & Kuizenga*, 1945; *Pabst et al.*, 1947), and in the anti-insulin test (*Grattan & Jensen*, 1940).

When preparing AC extracts for clinical use it is important that they should contain a certain amount of the two groups of corticosteroids. Unfortunately no specific test for the determination of the mineralocorticoids is at present available, and hence it is necessary to use one of the tests in group I and the sodium retention test of *Hartman et al.*; in addition it would seem desirable, as stressed by *Thayer* (1946), to test the activity of the glucocorticoids, since their content varies much more than that of the other factors.

THE URINARY EXCRETION OF CORTICOSTEROIDS

Normally the blood content of corticosteroids is so small as not to be demonstrable even with the most sensitive methods. In 1931, however, *Perla & Marmorston-Gottesmann* showed that a benzene extract from urine was able to increase the resistance of the adrenalectomized rat to histamine poisoning. This discovery was followed in the next few years by a large number of publications which showed that cortin is normally excreted in the urine, that its excretion may be increased or

reduced in certain pathological conditions, and that it may undergo physiological variations (*Grollman & Firor, 1933; Anderson & Haymaker, 1937, 1938; Weil & Browne, 1939, 1940, 1944; Dorfman et al., 1942, 1943; Horwitt et al., 1943; Schiller et al., 1943; Shipley et al., 1943*). An examination of the available literature shows that these pioneer studies give only few numerical data on the amounts excreted, and that the investigations have only been carried out on a few subjects.

With the adoption of the various chemical methods for the determination of these substances in the urine, however, findings in a large number of subjects have been reported during the last five years, especially in America. These included normal subjects as well as subjects with pathological conditions, and these studies suggest that the determination of the urinary excretion gives a fairly accurate indication of the adrenal cortical secretion of corticosteroids. *Vogl's* studies in particular have shown that only a small proportion of the amount actually produced is excreted in an active form, so that the amount excreted gives only a rough indication of the amount produced. Furthermore it should be stressed 1) that we do not, as yet, know all the details about the structure of the biologically active corticosteroid-like substances in the form in which they occur in the urine (*Lieberman et al., 1947; Mason & Sprague, 1948*), and 2) that in the determinations of the hormone by chemical methods the values obtained will often be too high as various inactive steroids are probably included.

Preparation of the urine extract:

In biological tests the purity of the final extract need not be particularly high, as the assay will be unsatisfactory only if the preparation is toxic to animals. While being collected the urine should, therefore, be stored in a cool place or be treated with chloroform as preservative. After completion of the collection, extraction should be done within 48 hours, otherwise the urine should be frozen, in which state it will probably keep for a very long time. In the case of a high litre

a 24-hour urine specimen is sufficient, otherwise a specimen collected over 48 hours is necessary. The urine is acidified to p_H 1 with H_2SO_4 , and it is then possible according to *Venning et al.* (1946) to extract an amount of biologically active material which is about twice that obtained for untreated urine. Extraction is carried out with ethylene dichloride or chloroform, $\frac{1}{4}$ vol. three times. The extract is evaporated in vacuo, and the temperature should never exceed $40-50^\circ C$, as the active substances are rather labile. The residue is taken up in chloroform, which is extracted with $N/10$ NaOH and water. It is then evaporated to dryness and stored in the cold ($-10^\circ C$) until the day of assay.

If the extract is to be used for the chemical determination of the corticosteroids, essentially the same procedure may be used, or chloroform-ether (1:4) may be used as the means of extraction (*Heard et al.*, 1946); since, however, the chemical methods most commonly used depend on the reducing power of one or more substituents in the molecule, it is necessary to carry out the extraction very carefully, to avoid all other reducing substances which might influence the results, and only use very clean glassware and very pure reagents. Usually much smaller amounts of urine will be sufficient. The chemical assay may be carried out on the crude extract, but with this procedure there is a risk that the values obtained may be too high; one possible reason for this is the presence of non-ketonic reducing substances.

For a further purification of the extracts the procedure described by *Venning et al.* (1944), and later, by *Talbot et al.* (1945) may be used:

Solution of the dry crude extract in benzene,
 extraction of the benzene with water several times,
 extraction of the water fraction with chloroform, removal of
 the chloroform by evaporation,
 partition of the residue with Girard's reagent T into the ke-
 tonic and non-ketonic material,
 chemical determination of the ketonic fraction.

These procedures are fairly elaborate and time consuming.

Thompsett & Oastler (1947), however, found only slightly higher, but constant values when the partition between benzene and water was omitted. *Talbot et al.* ran the four C₁₁-oxygenated corticosteroids through all the above procedures and obtained an average of 91 per cent in the ketonic fraction. Furthermore they showed that the more oxygenated biologically active corticosteroids passed quantitatively into the water while corticosterone and its 11-dehydro derivative was removed incompletely. DOC remains entirely in the benzene. By biological assay of the ketonic fraction, *Venning et al.* found an activity approaching that which one would expect in the case of a pure crystalline glucocorticoid; but as has already been noted, the chemical determination will often give slightly higher values.

Chemical methods for the determination of the corticosteroid-like substances in urine.

1. *Fieser, Fields & Lieberman* (1944) assayed certain steroids in the urine by means of the periodic acid oxidation reaction. In 1945 *Dobriner et al.* showed, however, that the method was unreliable for assaying the corticosteroids.
2. *Lowenstein, Corcoran & Page* (1946) modified the method, introducing a quantitative measurement of the formaldehyde liberated by the oxidation by which procedure 1 mol per oxygenated mol of corticosteroid is obtained. Oxygenation takes place at the primary alcoholic group at C₂₁.
3. *Daughaday, Jaffe & Williams* (1948) were able to use the method for the determination of the pure crystalline corticosteroids, but this method could not be applied to urinary extracts. These authors therefore introduced a distillation procedure, which eliminated the unspecific coloured substances present in the urine. To the distillate »chromotropic acid reagent« is added and the colour developed is compared colorimetrically with that obtained with known solutions of formaldehyde. Cortin is used as standard.

4. *Talbot, Saltzman, Wixom & Wolfe* (1945) used Nelson's modification of Folin & Wu's method for the determination of blood sugar to assay substances in the urine which are believed to correspond to those corticosteroids which have a ketonic or hydroxyl group at C_{11} , and which have a sugar-like or ketolic side chain and a hydroxyl group at C_{17} . The reaction is dependent on a reduction of cupric ion to a cuprous ion, which in turn reduces the arseno molybdic acid and develops a blue colour which is measured photometrically at 660 m μ . Amounts as low as 18 μ g. can be measured. Corticosterone is used as a standard.
5. *Heard, Sobel & Venning* (1946) developed a method which is based on the glucose-like reducing property of the primary α -ketol side chain at C_{17} and of the unsaturated α, β -3-keto group. The authors called these substances: »The neutral lipide-soluble substances of urine«. For the determination of the reduction property, Folin & Wu's phosphomolybdic acid reagent is used. The blue colour which develops is read in a photometer at 650 m μ . The results are expressed in terms of mg. DOC, which is used as standard.

Evaluation of methods for the determination of corticosteroids in the urine.

It is difficult to decide which of the methods described gives the fullest and most reliable information about the function of the adrenal cortex which, as already mentioned, possesses a variety of functions. There are good reasons for using the liver glycogen deposition test, since with this method we can measure substances of specific and high biological activity, which in all probability originate only in the adrenal cortex. Besides, they constitute what is perhaps the most important fraction of the corticosteroids and are of great importance to the organism: e. g. for protein and carbohydrate metabolism, for muscular activity and possibly other processes necessary for the maintenance of life. Moreover it would seem that they are essential for the resistance to »stress« and other external influences.

In many cases a chemical determination will undoubtedly be sufficient, but since the values obtained may, under certain circumstances, be too high, a simultaneous biological determination would at present seem desirable. The two last mentioned chemical methods (4. and 5.) are those which have been most commonly used, but it is difficult to venture an opinion as to the specificity of the methods before knowing which radicals take part in the chemical reaction.

It should be pointed out that in the determination of the neutral 17-ketosteroids in the urine, the biologically active corticosteroid-like substances are not included.

Urinary excretion in normal subjects.

In 1946 *Venning & Kazmin* reported the results of investigations made on a large number of normal subjects, in whom the amount excreted was determined biologically by means of their liver glycogen deposition test. The results are shown in table 1.

Table 1.

The figures in brackets below the results give the average result.

	Venning et al		Heard et al.		Lowenstein et al.	Talbot et al.
	glycogenic units/24 hrs.	ratio to normal	Unit: DOC mg/24 hrs.	ratio to normal	Unit: Cortin mg/24 hrs.	Unit 11-dehydrocorticosterone mg/24 hrs.
Normal men	40—85 (60)	1.0	1.1—2.1 (1.53)	1.0	} 0.5—0.8	} 0.10—0.45 (0.22)
Normal women	25—65 (41)		1.0—2.0 (1.34)			
Children 2.5 year	36	0.60	0.32	0.21		
3.0 —	42	0.71	0.47	0.31		
5.5 —	53	0.93	0.70	0.46		
7.0 —	58	0.98	0.79	0.52		

It is seen that the values for women are about one-third lower than those for men. Furthermore, it is interesting to note that while excretion at birth is very low, it rises fairly rapidly to reach the adult level at the 5th year, in contrast to

the 17-ketosteroid excretion, which only reaches correspondingly high levels much later in life (*Hamburger, 1948*).

For comparison the results in a number of normal subjects tested by *Heard, Sobel & Venning (1946)* by means of their chemical method are shown in the table. Here too the average values for the two sexes show a clear difference. The figures in children are somewhat lower than those found by the biological method. It should, however, be emphasized that only a few urines from children have been assayed and, that the chemical method possibly assays other steroids in addition to the glucocorticoids.

Finally, the values obtained by *Talbot et al. (1947)* and by *Lowenstein et al. (1946)* by their respective chemical methods have been given. They found no difference between the two sexes. *Shipley et al. (1946)* studied the urine of 7 normal subjects with the cold test and obtained values corresponding to 0.5—1.8 mg. of 11-dehydrocorticosterone in 24 hours, while in twelve subjects tested with the liver glycogen test, they found amounts corresponding to 0.2—0.8 mg. of 11-dehydrocorticosterone. The cold test is not comparable to the glycogen test, since it is possible that other corticosteroids may have an effect on it. The values are not directly comparable to those obtained by *Venning et al. (1946)*, because they used another standard, viz. 11-dehydro-17-hydroxycorticosterone. Finally it should be observed that the urine was extracted at p_H 5.5—6.5.

Diurnal fluctuations.

It has recently been shown that the excretion is at its lowest level during sleep, i. e. in the morning urine, and that it is higher in the forenoon than in the afternoon (*Pincus, 1947 a; Talbot et al., 1947*).

Stress.

A number of studies (*Venning et al., 1946; Browne et al., 1947; Pincus, 1947 b*) have been performed showing that the excretion rises after exertion or other »stress«. Thus *Venning et al.*, by examining the urine obtained from a group of sol-

diers before and after marching, found a three-fold rise in the glucocorticoidal activity.

Pregnancy.

While no variation in excretion has been found during the menstrual cycle, *Venning* (1946), in studies on 9 pregnant women, found a moderate rise in the values in the first third of pregnancy, followed by a return to normal values. From about the 150th day the values rise again and may attain a very high level. A simultaneous determination of the 17-ketosteroids showed only a very small, or no, rise. *Tobian Jr.* (1948) assayed the urinary excretion by means of the method devised by *Lowenstein et al.* and found values in normal pregnancy twice the normal, while the values rise furthermore in case of twins, toxemia and hypertension.

THE EXCRETION IN THE URINE IN PATHOLOGICAL CONDITIONS

Hypofunction of the adrenal cortex.

This, as is well known, may be caused by a lesion in the adrenal cortex, due frequently to tuberculosis, but may also be produced by a lesion in the anterior lobe of the pituitary gland and possibly in the hypothalamus, bringing about a reduced production of the corticotrophic hormone, which results in atrophy of the adrenal cortex. In Addison's disease low values are generally found, even though a few patients in good general health will show values within the normal limits. In AC-insufficiency with a hypophyseal aetiology, very low values are almost always found. Hence the determination of the corticosteroids can be used to differentiate this condition from anorexia nervosa, in which the excretion is normal.

Reduced values are also frequently found in hypofunction of the thyroid gland. In these cases there is a fairly good agreement with the excretion of 17-ketosteroids.

Hyperfunction of the adrenal cortex.

Increased excretion of corticosteroids is found in cases in which unspecific pathological changes are present. They can all be correlated with *Selye's* theories on the adaptation syndrome. These conditions include infections, operations, burns and other serious injuries to the body (*Shipley et al.*, 1946; *Browne & Venning*, 1947). The excretion generally rises rapidly, most frequently within the first 24 hours, and the rise may persist for a very long time, not uncommonly until healing is complete. Thus, *Talbot et al.* (1947) found that with an extensive burn there were high values which persisted for as long time as 2 months. In these cases the excretion of 17-ketosteroids is usually normal.

Among the diseases involving hyperfunction of the adrenal cortex, two distinct types may be differentiated, viz. Cushing's syndrome and the adrenogenital syndrome (*Cahill*, 1944; *Dahl-Iversen & Hojensgaard*, 1947; *Wilkins*, 1948; *Soffer*, 1948). There are, in addition many types transitional between the two and, as emphasized particularly by *Kepler*, types of the disease in which only one symptom is present. The studies of *Venning et al.* (1947) suggest that in Cushing's syndrome, an overproduction of the glucocorticoids is the decisive factor. They found a greatly increased excretion of these steroids, while the excretion of 17-ketosteroids was normal or slightly increased. Studies on a large number of subjects are, however, required in order to confirm these findings. In the adrenogenital syndrome the position seems to be the reverse, which is in agreement with the hypothesis that the decisive factor here is a greatly increased production of steroids with an androgenic effect; high 17-ketosteroid values but normal or slightly increased corticosteroid values are thus found (*Dobriner et al.* 1942; *Patterson et al.*, 1942; *Callow & Crooke*, 1944; *Mason & Kepler*, 1945; *Venning*, 1948; *Wilkins*, 1948).

Another problem of great practical significance in clinical medicine, is the differentiation between tumour and hyperplasia of the adrenal cortex. The studies carried out up til

now seem to indicate that the corticosteroid determination is of no real significance.

In table 2, an attempt has been made to list the significant values so far obtained; the scattered results on individual

Table 2.

The figures in brackets above the results show the total number of subjects examined.

Disease	Venning et al. glycogenic units/24 hrs.	Daughaday et al. (Unit: Cortin) mg/24 hrs. (urine acidified to: pH 1.7) Normal values: 1.0-1.6 mg	Talbot et al. (Unit: 11-dehydrocortico- sterone) mg/24 hrs. (unacidified urine) Normal values: 0.10-0.44 mg
hypofunction of AC.	(4) < 10	(6) 0.3-0.65	(17) 0.02-0.29
hypopitu- itarism.	(4) < 10	(?) 0.5-0.8	(7) 0.04-0.29 6 of 7 < 0.18
anorexia nervosa	(3) 17-36		
hypothyro- idism.		diminished	(2) 0.07-0.13
Cushing's syndrome	(5) 137-700	(?) +++	(12) 0.6-12.0
adrenogenital syndrome	(?) norm.or (+)	(?) (+)	(5) 0.24-0.57
hirsutism. (simplex)	(4) 39-65		(3) 0.23-0.32

subjects found in the literature have been left out. Some investigators have subdivided the adrenogenital syndrome; this subdivision is, however, omitted from the table, which is limited to the excretion of corticosteroids.

Simple hirsutism has been included, being undoubtedly a border line pathological condition (cf. monosymptomatic forms).

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ANNOUNCEMENTS

from the Endocrinological Societies

DUTCH SOCIETY FOR ENDOCRINOLOGY

3. Meeting, March 3, 1948, Amsterdam.

- de Vink, L. P. H. J.* (Amsterdam): a. Dystrophia adiposo-genitalis.
b. Some cases of amenorrhea.
- J. A. Wijsenbeek* (Amsterdam): Difficulties in the treatment of amenorrhea.
- G. A. Lindeboom* (Amsterdam): Irreparable damage after administration of normal amounts of insulin.
- H. J. Viersma & O. M. de Vaal* (Amsterdam): Paroxysmal hypertension caused by a phaeochromocytoma.
- J. Groen* (Amsterdam): Oestrogen-treatment of carcinoma of the prostate with bone metastases.
- H. Pelser, E. G. Godfried & J. Groen* (Amsterdam): Treatment of bone metastases of carcinoma mammae with testosterone propionate.
- E. Dingemanse & L. G. Huis in't Veld* (Amsterdam): Excretion diagrams of 17-ketosteroids in patients with tumours of the adrenal cortex.

4. Meeting, May 8, 1948, Amsterdam.

- G. Lever* (Utrecht): Histological evaluation of thyroid function.
- B. Greene* (London): The treatment of thyrotoxicosis in England.
- J. Mahaux* (Brussels): Thyro-hypophyseal interrelations in experimental and clinical pathology.

5. Meeting, Nov. 27, 1948, Amsterdam.

- A. J. M. Holmer* (Leiden): The differential diagnosis of amenorrhea.
- J. B. Stolle* (Tilburg): Endocrine disturbances due to chronic inanition.
- J. C. A. Mighorst* (Utrecht): The biological basis of the Konsuloff-reaction.
- A. Querido* (Leiden): On hypometabolism.

SWEDISH SOCIETY FOR ENDOCRINOLOGY

Meeting, November 29, 1948.

- E. Malmberg*: Death in agranulocytosis due to treatment with methylthiouracil in thyrotoxicosis. Report of a case.
- C. G. Bergstrand*: Pubertas praecox in a boy operated upon for hydrocephalus.
- J. P. Naeslund*: Influence of hormones on the genital development in hen's egg.
- F. Paulsen*: The chemistry of the hypophyseal hormones.

DANISH SOCIETY FOR ENDOCRINOLOGY

12. Meeting, Dec. 17, 1948. Domus Medica, Copenhagen.

- Jens Vilh. Thorborg*: On the influence of oestrogenic hormones on the male accessory genital system.
- Aa. Theil Nielsen*: Colorimetric assay of testosterone and some other steroid hormones by means of a modified Kober reaction.
- Chr. Hamburger*: »Micro-methods« for the determination of 17-ketosteroids in urine.

From the Zoological Laboratory, Department of Endocrinology
(Professor G. J. van Oordt, Ph. D.) and the University Hospital,
Department of Gynecology (Professor W. P. Plate, M. D.),
Utrecht.

THE VALUE OF THE MELANOPHORE REACTION IN *RANA ESCULENTA* (*KONSULOFF*-REACTION) AS A PREGNANCY TEST

BY

J. C. A. MIGHORST, L. A. M. STOLTE, P. H. M. de ROO
and F. CREUTZBERG

I. INTRODUCTION

In 1919 it was shown by *P. E. Smith* that in Amphibians, melanophore dispersal is caused by a hormone secreted in the intermediate lobe of the pituitary. In view of this observation, hypophysectomized frogs or isolated pieces of frog's skin were used to test the presence of melanophore hormone (M. H.) in all kinds of organ extracts and body fluids, and it was found that this hormone is secreted not only by the pituitary of the Vertebrates, possessing skin chromatophores, but also by the pituitary of mammals, in which this kind of pigment cell is lacking. However, the biological significance of this hormone in mammals is still obscure. *Jores & Caesar* (1935) associate it with the eyes' dark-adaptation, whereas *Zondek* (1935) attributes an antidiuretic action to it.

The occurrence of M. H. in men led to several investigations dealing with the problem, and particularly as to whether the M. H.-concentration is modified under different circumstances.

Küstner & Biehle (1927, 1928) were of the opinion that in the serum of pregnant, parturient and puerperal women changes in the concentration of M. H. take place, but this was, however, denied by *Jores & Helbron* (1933) and *Zondek* (1935).

On the other hand, *Collin & Drouet* (1933) claim that the urine of patients with pituitary tumors, hyperthyroidism, retinal haemorrhage, chloride-retention and also the urine of menstruating women possesses a melanophore dispersing action.

As these and similar observations were made on non-hypophysectomized frogs, they may be explained by a stimulating action of the urine on the pituitary, which affects the secretion of M. H. and thus produces an indirect melanophore reaction. Hence exact investigations about the action of M. H. should be carried out in hypophysectomized frogs.

In 1934 *Konsuloff* published his first results, obtained with hypophysectomized frogs, in which human urine was injected into the dorsal lymph sac. His conclusion was that melanophore dispersal takes place only with pregnancy urine. According to him the reaction is a rapid and reliable pregnancy test. *Jores* (1936), however, claimed that the reaction is not specific and that many substances, present in urine, stimulate the melanophores directly, resulting in their dispersal.

Shen (1939) found that ether and chloroform among others have a direct action on the melanophores, whereas other substances like barbiturates are only capable of stimulating the frog pituitary.

In 1946 *de Bourgraaf & Dingemanse* found that the *Konsuloff*-reaction is reliable, provided it is performed under conditions similar to those we have also used (cf. p. 100). Independently and almost simultaneously *Servantie, Cambàr, Moretti & Bonnal* (1947) also re-introduced the *Konsuloff*-reaction as a pregnancy test. Whereas the colouring of the whole frog is taken as an index by *de Bourgraaf & Dingemanse*, the French workers pay attention to the effect on the melanophores themselves, for which a melanophore index is given.

In view of these statements we have investigated the prac-

tical usefulness of the *Konsuloff*-reaction. In addition we have tried to determine whether the melanophore dispersing substances, present in human pregnancy urine, are identical with M.H., with chorionic gonadotrophin or possibly with other substances, which have a direct stimulating effect on the melanophores (cf. *Shen*, 1939). In connection with this, we have thrown some light on the mechanism of the *Konsuloff*-reaction as a pregnancy test, as compared with the *Aschheim-Zondek*-reaction.

II. MATERIAL AND METHODS

For the *Konsuloff*-reaction only specimens of *Rana esculenta* were used with a bright grassgreen coloured back and with yellowgreen patches on the inside of the thigh. After hypophysectomy these frogs assume a soft yellowgreen colour, whereas the thigh patches turn into a vivid yellow.

Hypophysectomy was performed, with small modifications, by the method described by *Servantie et al.* (1947). The cartilage situated under the base of the parasphenoidal bone is not removed, but incised semi-circularly and turned backwards. The entire pituitary is removed with the help of a small pipette and a narrow rubber tube, by means of the well-adjustable sucking force of the operator's mouth. Attention should be paid to the fact that the anterior lobe is easily torn from the rest of the pituitary. All bleeding must be avoided. After the operation the above-mentioned cartilage is turned back to its original position so that the cerebral cavity is closed.

We have kept our hypophysectomized frogs alive in the laboratory for several months. If the operation is performed during the oestrous period the mortality is very high; during the summer and autumn only very few animals died following the operation. The experimental animals were kept in aquaria, in which the temperature never exceeded 15° C and of which the bottom was partly dry and partly covered with water. The frogs were fed on mealworms, which were eaten avidly.

The conditions under which the melanophore reactions were performed, were more carefully worked out than those of *de Bourgraaf & Dingemanse* (1946). They are as follows:

1. The experimental frogs must be completely yellowgreen; only then can one be certain that they are totally hypophysectomized; moreover, the spherical state of the melanophores can be controlled by microscopic examination of the animal's webs.
2. The experimental frogs must be treated with pregnancy urine from time to time, as sometimes the melanophores of frogs, which have been used repeatedly over a long period, do not react.
3. The investigator must have some experience in evaluating the results of the test. In some cases normal urines show a very slight reaction, while the reaction to a pregnancy urine may be weak; only experienced workers are able to assess the results accurately in these cases.
4. For each test 3 frogs, having about the same colour pattern, must be used; two are injected with the urine to be investigated, and one, the darkest of the three, acts as a control.
5. Different volumes of urine must be injected, varying according to the size of the frog; they were for

frogs under 10 grams	1	ml.
» between 10—20 grams	1.5	»
» » 20—30 »	2	»
» » 30—40 »	2.5	»

The injection is given subcutaneously into the dorsal lymph sac about 1 cm. from the cloacal opening. During the reaction the frogs are kept in a dry place.

6. The optimal temperature in which the animals react is 22—24° C. To adapt their basal metabolism the frogs must be exposed to that temperature for several hours before the test is performed.

7. Usually the reaction is obvious 1 hour after the injection, but a definite conclusion must not be drawn until $1\frac{1}{2}$ h. have passed.
8. The colour-judgment must take place in diffuse daylight, not in artificial light. Before the test is carried out the colour of the frogs must be accurately compared; the upper parts of the hindlegs and the back must be particularly considered.
9. The reaction is positive, if the colour darkens first over the thigh and then eventually over the back. By that time the yellow patches on the inside of the thigh have disappeared. The appearance of a contrasting colour of the thigh with the back is a good criterion for a positive reaction; but the reaction is also positive if the colour darkens over the whole body. The reaction is doubtful if the darkening of the frog's body is only slight and does not show the stages described above.

III. THE CLINICAL VALUE OF THE MELANOPHORE REACTION

A correct judgment of the melanophore reaction as a pregnancy test is only possible if the positive or negative result can be compared with the clinical condition of the patient. Therefore we have only taken into consideration those results in which the patient could be fully controlled.

We have considered:

1. a positive reaction as correct, and a negative reaction as incorrect if:
 - a. positive pregnancy signs were present,
 - b. evidence of pregnancy (placental tissue, chorionic villi) was obtained after curettage,
 - c. an ectopic pregnancy was found at laparotomy (chorionic villi),

2. a negative reaction as correct, and a positive reaction as incorrect if:
 - a. the menstrual cycle proceeded normally,
 - b. no chorionic villi were found after curettage,
 - c. no chorionic villi were found after laparotomy (serial sections),
 - d. if pregnancy was out of the question (e. g. after hysterectomy).

The result is uncertain if after a short period of amenorrhea a slightly increased bleeding appears. In this case an unperceived abortion may have occurred.

According to this scheme, 83 urines were tested in a preliminary investigation. As a rule the clinical diagnosis was known to us before the test was performed. 72 urines, 65 of which belonged to normal pregnancy-cases, gave a positive reaction. One positive urine came from a man with a basophilic hypophyseal adenoma; after having been operated upon, his reaction became negative. Six urines were from patients with abortion or ectopic pregnancy.

Among the 11 negative reactions one proved to be incorrect; in this case the urine was from a patient who had tried to practice abortus provocatus, 15 days before the melanophore reaction was performed. Ten days later the test was repeated with a positive result; later on pregnancy was obvious.

We have also made a few observations to determine the day of the gestation period on which the melanophore reaction becomes positive, and how many days after parturition the reaction becomes negative:

The urine of a woman with a normal menstrual cycle of approximately 29 days was tested regularly. Before cessation of menses two reactions were carried out, both with negative result, this being also the case on the 29th day. From that day onwards daily examinations were made. The first positive result was found on the 33rd day after the last menses.

In 4 cases urines were investigated beginning on the day

of parturition. The following day the result was still positive; in one case the urine was negative 3 days after parturition, but two days later all the urines showed a negative result.

The favourable results of these preliminary investigations led us to further clinical applications and to an attempt to establish the nature of the principle responsible for the *Konsuloff*-reaction.

The *Aschheim-Zondek* pregnancy test (A.-Z.) and the *Konsuloff*-reaction (K.), carried out with a sample of the same urines, were done by one of the present authors in 525 cases. In these cases clinical diagnosis was not known before the tests were performed. The results are shown in table 1. (A doubtful K.-reaction is that found in frogs which on the whole darken a little, but in which the darkening of the thigh colour is not distinct. A.-Z.^I means that a reaction of the mouse-uterus took place, whereas blood-spots or corpora lutea were absent in the ovaries; A.-Z.^{II} and A.-Z.^{III} mean that a reaction with blood-spots or corpora lutea was present.)

Table 1.

Results of the *Konsuloff*-reactions compared with those of the *Aschheim-Zondek*-reactions.

	K +	K —	K ?	total
A.-Z. ^I +	23	29	12	64
A.-Z. ^{II, III}	200	14	11	225
A.-Z. —	13	195	28	236
total	236	238	51	525

From table 1 it follows that in 424 cases a definite agreement between both reactions was found (200 A.-Z. +, K. +; 195 A.-Z. —, K. —; 29 A.-Z.^I +; K. —); completely contradictory results occurred in 27 cases or 5.3 per cent (13 A.-Z. —, K. +; 14 A.-Z. +, K. —). In 23 cases an A.-Z.^I-reaction (which is not considered definite proof of pregnancy) was found together with a positive K. In these cases discrepancy is possible, though not certain. However, we are not justified in

drawing the conclusion from these figures that the *Konsuloff*-reaction gave incorrect results in 5 per cent of the cases. This would only be the case if the urines with which the A.-Z.-reaction was found positive and the K.-reaction negative, were solely obtained from cases with a normal, undisturbed pregnancy and not e. g. from cases of missed abortion or ectopic pregnancy. Conversely (A.-Z. —, K. +) the possibility that in early pregnancies the A.-Z.-reaction is not yet positive should be excluded.

As in many cases clinical control was not available, we cannot determine from these figures how many times the *Konsuloff*-reaction gave an incorrect result. The percentage is certainly far below 5 per cent.

From table 1 it follows further that the K.-reaction was doubtful in 11 cases, in which the A.-Z.-reaction was positive, and in 28 cases in which the A.-Z.-reaction was negative.

The meaning and value of a dubious K.-reaction is not *a priori* clear. The quantity of the melanophore dispersing principle, present in urine, may not be sufficient to give a fully positive pregnancy reaction. On the other hand toxic urine substances may have a weak positive action on the melanophores. This is the case also, when the frogs die. In this connection *Servantie et al.* (1947) refer to the »dilatation préa-gonique de mélanophores«. Hence positive reactions of frogs, which die some time after the experiment, are not reliable; they have been omitted from table 1.

The clinical finding has to be taken into consideration, but an A.-Z.-reaction is usually an indication that the test should be repeated. Among the 525 cases (table 1) this occurred 64 times. In our view a doubtful K.-reaction has the same clinical, though not the same biological value as an A.-Z.-reaction. Such a doubtful result was obtained in 55 cases.

As some discrepancy existed between the results of the A.-Z.- and K.-reactions, 91 clinical cases, taken from the 525 cases of table 1, were investigated. In these 91 cases 107 K.- and A.-Z.-reactions were done, with a sample of the same urines (table 2). Special attention was paid to the question

of whether the pregnancy was intact or disturbed. A positive A.-Z. is merely a proof of the presence of active, gonadotrophin producing chorionic tissue. It does not answer the question whether the foetus is dead or alive. Moreover, other investigations gave us the impression that the K.-reaction is not dependent on chorionic function and therefore the discrepancy between both these reactions might be correlated with some other phenomena relating to pregnancy or its sequelae.

Table 2.

Results of the special clinical cases, mentioned p. 105—107.

	K +	K —	K ?	Total
A.-Z. ^I +	6	2	4	12
A.-Z. ^{II, III} +	30	2	6	38
A.-Z. —	5	41	11	57
Total	41	45	21	107

From table 2 it follows that the K.-reaction gave more positive and less negative results than the A.-Z.-reaction. As a matter of fact the number of doubtful reactions is relatively larger in these 107 cases, which, however, form part of the 525 cases, summarized in table 1. This is obviously due to the fact that among these cases many disturbed and early pregnancies are present. Of course neither doubtful K.-reactions nor A.-Z.^I-reactions are here considered as proof of pregnancy.

In 71 cases (30 A.-Z. +, K. +; 41 A.-Z. —, K. —) the result was confirmed by clinical diagnosis. In 3 cases of the group A.-Z. —, K. —, chorionic villi, found after curettage, showed post-pregnancy indications.

In 5 cases A.-Z. —, K. + two pregnancies were found, i. e.

1. a pregnancy of 6 weeks, in which the A.-Z. became positive later on,
2. a tubal pregnancy (chorionic villi), in which the A.-Z. remained negative repeatedly.

In 2 more cases (A.-Z. —, K. +) the K.-reaction indicated a pregnancy incorrectly, i. e.

1. in a patient with a corpus luteum cyst (pseudo-pregnancy). As curettage was not performed, proof that chorionic villi were absent was not obtained. Clinical observation gave no indication whatever of the existence of an intra-uterine pregnancy.
2. in a patient with folliculus persistens, who was once injected intramuscularly with 10 mg. progesterone.

The 5th case was one of missed abortion, a disturbed pregnancy in which the A.-Z. —, as well as the K. + could be called correct.

In the 6 cases A.-Z.^I +, K. +, the A.-Z.-reaction had an incorrect result 3 times:

1. on the 66th day after the last menses (on the 80th A.-Z. was also +),
2. on the 48th day after the last menses (on the 66th A.-Z. was also +),
3. on the 42nd day after the last menses (on the 61st A.-Z. was also +).

In one case of habitual abortion there was primary fetal death. In two cases proof of pregnancy could not be given, i. e.

1. in a patient of suspected ectopic pregnancy, who did not react to a resorption therapy. On operation a haematosalpinx was found, in which, however, no chorionic villi were seen (no serial sections),
2. in a patient, who was curetted for fluxus 2 weeks after the reaction had been performed, an endometritis was found; chorionic villi and secretion phenomena, however, were absent (clinical diagnosis: »unperceived abortion«).

In the 2 cases A.-Z. +, K. —, the result of the K.-reaction was incorrect i. e.

1. on the 37th day after the last menses [later on K. was + (intact pregnancy)],
2. in a case of diluted urine from hydatidiform mole.

Among the 6 cases A.-Z. +, K ? one, a case of a mother nursing her child, there was an intact pregnancy of about 8 weeks. In the other cases the pregnancy was either disturbed (habitual abortion following) or certainly disturbed (tubal abortion, threatening abortion, infected ectopic pregnancy, diluted urine from hydatidiform mole).

Of the 4 cases A.-Z.¹ +, K. ? one related to an intact pregnancy (low concentration of urine?); in one case pregnancy was absent, in two other cases pregnancy, if present, was certainly seriously disturbed.

In 7 of the 11 cases A.-Z. —, K. ? pregnancy was absent, and in 4 other cases pregnancy was, if present, completely disturbed. Therefore a doubtful melanophore reaction should not always be taken as a negative reaction (2 intact and 7 presumably or certainly non-intact pregnancies). Consequently atypical *Konsuloff*-reactions must be interpreted as non-positive for pregnancy and must be repeated.

Summarizing we have seen that among 107 experiments the A.-Z.-reaction never had an incorrect positive result, whereas the positive K.-reaction indicated a pregnancy incorrectly at least once; on the other hand, the A.-Z.-reaction failed to demonstrate an intact pregnancy in 5 cases and the *Konsuloff*-reaction in 3 cases. The A.-Z.-reaction »warned« 4 times, whereas a doubtful K.-reaction »warned« twice. That the percentage of failures of the A.-Z.-reaction is rather high, is certainly due to the special nature of the material: many cases of disturbed and early pregnancies.

IV. THE NATURE OF THE PRINCIPLE RESPONSIBLE FOR THE *KONSULOFF*-REACTION

Collin & Drouet (1933) and *Lambillon & Lejeune* (1938) using non-hypophysectomized, lightcoloured frogs, were able to evoke melanophore dispersal with the urine of patients suffering from very different diseases. However, we found negative K.-reactions as well as A.-Z.-reactions in cases of dermoid

cysts, endometriosis ovarii, follicle-cysts, myomatosis of the uterus, appendectomy, double-sided hydrosalpinx, ovarian cysts with haematoma, salpingitis, chondrometriosi, lactation-, hormonal and physis amenorrhoea, hypomenorrhoea, climacteric and preclimacteric bleeding.

Umezawa (1935), working with normal non-hypophysectomized frogs, observed a melanophore dispersal after the injection of urine of cancer patients, and proposed to use this reaction for the diagnosis of cancer. It is therefore worth mentioning that we got fully negative results in two cases of stomach carcinoma, in two cases of seminoma testis, and in cases of prostate carcinoma, mammary carcinoma, carcinoma of the ovary and carcinoma of the cervix uteri.

From the literature dealing with melanophore dispersal, it may be concluded that in normal urines substances are found with a melanophore dispersing function. But this does not occur often enough to make the K.-reaction unreliable. *Shen* (1939) pointed out that barbiturates produced melanophore dispersal in normal frogs; our patients who had been given barbiturates, however, showed a negative K.-reaction. In patients with a hormonal disturbances the K.-reaction was generally negative; on the other hand, it proved to be positive in cases of hydatidiform mole and chorion-epithelioma (also of the testis), of basophilic adenoma of the pituitary in a man (after removal of the tumor the reaction became negative!) and of Graves' disease in a woman with a negative A.-Z.-reaction (basal metabolism: + 47). But in a case of hypophyseal myxoedema, of *Cushing's* disease in a man and in a woman, of Graves' disease in a man, and of mammary carcinoma in a woman, treated with high doses of methyltestosterone, the K.-reaction was negative.

In addition to pregnancy urine and urine of patients suffering from hydatidiform mole and chorion-epithelioma, melanophore dispersal could be produced in some cases of hormonal disturbance without pregnancy.

Unlike the urine of a pregnant Java-monkey, the urines and sera of pregnant cows, horses, frogs, guinea-pigs and dogs did

not evoke melanophore dispersal. Neither did watery extracts of endocrine organs of these mammals, with the exception of the pituitary.

Moreover, we stated that with very few exceptions the quantity of the dispersing principle is increased only in the serum and urine of pregnant women. Urine of a woman in parturition reacted positively; her serum, however, had only a weak positive reaction, whereas her cerebro-spinal fluid gave a negative reaction, as did the cerebro-spinal fluid of a patient with tubal abortion and of a patient with a corpus luteum cyst. Amniotic fluid (without urine), the first urine of the newborn child and serum of the umbilical cord as well as fluid from follicles, corpora lutea, a corpus luteum cyst and placental-tissue, oestrone, oestradiol, progesterone and F. S. H. gave a negative reaction. In the pituitary of a still-born child a large quantity of melanophore dispersing hormone was found. Ambinon (a thyro-gonadotrophin) and piton (oxytocin) gave a positive reaction as did pregnyl (a gonadotrophin prepared from pregnancy urine). The latter statement may be explained by the fact that the purification of pregnyl does not involve the separation of the melanophore dispersing principle from it.

From all these facts it may be concluded that the hormone, present in serum and urine, is of hypophyseal origin. Also in favour of this view is the finding that *Konsuloff*-reactions were obtained in cases in which chorionic gonadotrophin could not be present, i. e. with the urine of a male patient with a hypophyseal basophilic adenoma, of a female non-pregnant patient with an increased basal metabolism, of a patient with a corpus luteum cyst (A.-Z. —) and of a patient with persistent follicles (to whom 10 mg. progesterone had been administered intramuscularly). The supposition that only during pregnancy does the pituitary form hormones with melanophore dispersing function, and that then the melanophore hormone is formed in larger quantities and excreted in the urine, seems reasonable. Moreover, a melanophore dispersing

principle is also found in the urine of patients with an increased progesterone level (*cf.* p. 106).

That the results of the K.- and A.-Z.-reactions do not always agree, does not mean that they are caused by different principles. If both reactions were based on the action of chorionic gonadotrophin, it is obvious that the sensitivity to this hormone is different in both experimental animals, i. e. the mouse and the frog. But as the K.-reaction is positive in some cases, while the A.-Z.-reaction is positive in others, the only possible conclusion seems to be that these reactions cannot be due to one principle only, but must be due to two different principles and that therefore the K.-reaction cannot be dependent on the action of chorionic gonadotrophin. However, it is still possible that in cases, in which both reactions have a different result, the minimal effective concentration of only one principle is present, which acts within the variability of sensitivity of the experimental animals. In this case one principle could produce an A.-Z. +, K. — reaction in one, and an A.-Z. —, K. + reaction in another experiment. As we have seen, urines of early and of disturbed pregnancies (abortion), in which the gonadotrophin level can be still low, give positive results sometimes in the mouse and in other instances in the frog. But the fact that using diluted urines from cases of

Table 3.

Dilution-rates above the threshold value, in which the A.-Z.-reaction would become negative, and under the threshold value, in which the K.-reaction is positive.

Patient	A.-Z.-reaction	K.-reaction
A hydatidiform mole-pregnancy	1:400 +	1:80 —
B hydatidiform mole-remnants present	1:25 +	1:25 —
C hydatidiform mole-pregnancy	1:100 +	1:100 —
D chorionepithelioma	1:100 +	1:100 —
do., repeated after one week	1:100 +	1:100 —
E hydatidiform mole-pregnancy	1:200 +	1:200 +

hydatidiform mole, the A.-Z.-reaction always remains definitely positive, whereas the K.-reaction is already negative (table 3), and that in low concentrations of chorionic gonadotrophin during early and disturbed pregnancies, gestation is demonstrated sometimes by the K.- and in other instances by the A.-Z.-reaction, we may conclude once again that chorionic gonadotrophin does not bring about the K.-reaction and that the differences between both reactions are not dependent on the minimal effective concentration of one principle, acting within the variability of sensitivity of the experimental animals.

We will now lay stress on the observation, already mentioned on p. 106 according to which a positive K.-reaction could be obtained with the urine of a non-pregnant amenorrhoeic patient, who was injected with 10 mg. progesterone and with the urine of a patient with a corpus luteum cyst. As this result may point to an increased melanophore dispersing hormone-secretion in the pituitary, possibly produced by a high progesterone concentration, we have treated 10 other amenorrhoeic patients with progesterone. Eleven patients received 30 mg. of progesterone on the first day and 20 mg. on the second day. The K.-reaction was done regularly; days on which this reaction was performed are indicated with a positive or negative sign in table 4.

Most of the reactions were negative or doubtfully positive. Because the dosage might have been too small, patient 1, as well as a non-amenorrhoeic patient (nr. 12) got 30 mg. progesterone on 3 successive days, but the effect did not increase.

Summarizing we are of opinion that the K.-reaction is not caused by chorionic gonadotrophin (as is the case in the A.-Z.-reaction) but by the hypophyseal melanophore dispersing hormone and that perhaps the increasing progesterone concentration found during pregnancy stimulates secretion of the melanophore hormone in the woman's pituitary.

Our experiments on the influence of progesterone in amenorrhoeic patients are not fully conclusive, but together with

Table 4.

Results of the *Konsuloff*-reaction after injection of progesterone in amenorrhoeic patients.

Patient	1st	2nd	3rd	4th	5th	6th	7th
1	30 —	20 —	—		(+)	+	
2	30 —	20 —		—			
3	30 —	20 —	—	—	—	—	—
4	30 —	20 —	—				
5	30 —	20 —			—	—	
6	30	20	—	—			
7	30	20 (+)	—	—	—		
8	30 —	20	—	—	—		
9	30 —	20	(+)	(+)	✓		
10	30 —	20	—	—			
11	30 —	20 —	(+)	—	—	—	—
1	30 —	30 —	30 (+)	(+)	—	—	—
12	30 —	30 —	30 —	—	—	(+)	(+)

our clinical data, they are in favour of the view that progesterone stimulates the formation of melanophore hormone in the human pituitary.

The last question to be dealt with concerns the problem as to why the melanophore dispersing hormone is present only in urine of primates.

It is well-known that in men the intermediate lobe of the pituitary is only represented by an indistinct zona intermedia (Romeis, p. 290).

According to *Benda* (1932) and *Plaut* (1936) the function of the intermediate lobe in primates is taken over by the anterior lobe. *Roth* (1932) and *Jores & Glogner* (1933) claim that the melanophore hormone is formed by the basophilic cells of this lobe; according to *Jores & Glogner* (1933) the concentration of melanophore hormone was increased in a pituitary extract of a patient, suffering from a hypophyseal basophilic adenoma. On the other hand, it is accepted that the gonadotrophic hormone of the anterior lobe is also formed in basophilic cells. Moreover, we know that in mammals with a distinct intermediate lobe the melanophore hormone is not excreted in the urine during pregnancy. In primates, however, where the function of the intermediate lobe is taken over by the anterior lobe, this excretion takes place. Therefore it is not improbable that this phenomenon, only found in primates, is correlated with the fact that during pregnancy the anterior lobe has an increased hormone production, and that therefore the melanophore dispersing hormone, possibly stimulated by progesterone, is excreted only in the pregnancy urine of primates.

SUMMARY

1. The *Konsuloff*-reaction (K.) is, with certain precautions, a reliable pregnancy test.
2. Discrepancies between the K.-reaction and the *Aschheim-Zondek*-reaction (A.-Z.) carried out with the same urines,

were analyzed and correlated with the clinical data. The reliability of both reactions is about the same.

3. The K.-reaction is probably not caused by chorionic gonadotrophin (as is the A.-Z.-reaction), but by hypophyseal melanophore hormone, which during pregnancy is excreted in an increased concentration. This occurs only in primates.
4. Since progesterone administration is followed by an increased melanophore hormone excretion, it is suggested that progesterone stimulates the formation of the melanophore hormone in the pituitary.
5. As in primates, unlike other mammals, which possess a distinct intermediate lobe, the melanophore hormone formation occurs in the basophiles of the anterior lobe of the pituitary, the increased melanophore hormone excretion in urine is thought to be the result of the increased hormone formation in the anterior lobe of the pituitary during pregnancy.

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Addendum

The assumption that 1. the A.-Z.- and K.-reactions are not based on the same principle and 2. the corpus luteum is functionally related with the mechanism of the Konsuloff-reaction could be supported in the following way.

A 49 years old woman, suffering from menorrhagia, was injected on the 5th, 6th and 7th day after the beginning of

the bleeding with a dose of 20.000 R.U. (in total 60.000 R.U.) of pure gonadotrophin¹). On the 6th and 7th day the A.-Z.^{II-III}-reaction was positive, whereas the frogs became only slightly darker over the whole body (weak K.-reaction).

The same chorionic gonadotrophin, injected in a hypophysectomized frog, was totally negative, whereas a less pure preparation gave a positive reaction.

After laparotomy, executed 6 days after the onset of the bleeding, a corpus luteum cyst was found.

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¹) Pregnyl, prepared from pregnancy urine, presented to us by the Direction of Organon N. V., Oss.

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THE EFFECT OF GONADECTOMY, PREGNANCY
AND ADMINISTRATION OF OESTRADIOL ON
THE LYMPHATIC TISSUE OF THE SPLEEN
IN FEMALE GUINEA PIGS*)

BY

BENGT H. PERSSON

INTRODUCTION

The marked physiological variability of the lymphatic tissue of the spleen has been described by a number of investigators. This variability appears, moreover, to be a characteristic common to all lymphatic tissue, and is considered to depend both on its lymphocytopoietic activity and on its reaction to various irritants within the organism.

The relation between age and the amount of white splenic pulp has been confirmed by several workers, and the significance of the period of sexual maturity, as the time when the amount of white pulp reaches a maximum has been demonstrated, but the state of the gonadal activity has not yet been considered in connection with this problem. Clinical and experimental investigations have, however, suggested that

*) Aided by a grant from Svenska Sällskapet för Medicinsk Forskning (Swedish Society for Medical Research).

there is a relation between the gonads and the spleen as a whole, but the role of the white pulp in this has not been elucidated.

Without entering into the question of the function of the white splenic pulp, I nevertheless consider that it would be of some interest to study quantitatively its variations under the influence of sex hormones. This might show whether the variations are affected by the gonads.

SURVEY OF THE LITERATURE

Hellman (1913—14) showed on the rabbit that the lymphatic tissue of the spleen increases in amount until shortly after sexual maturity, and then undergoes a more or less pronounced involution. He also noticed that the lymphatic nodules (the Malphigian corpuscles) appear to occupy a unique position since their development reaches its maximum earlier than the rest of the white pulp.

The specific effect of the oestrogens on the lymphatic tissue of the spleen has not been investigated. In the studies on the adaptation syndrome *Selye* (1939, 1946) and other investigators have however called attention to the effect of *oestrogen overdosage* with atrophy of white splenic pulp. *Korenchevsky et al.* (1935, 1939) and other workers have investigated the effect of oestrogenic hormones on the weight of the spleen, but no uniform results were obtained.

The effect of ovariectomy is studied by *Masui et al.* (1926), *Cirillo et al.* (1933) and *Freudenberger et al.* (1935). These workers found an increase in the weight of the spleen after ovariectomy, but made no mention of the amount of the white pulp present in it.

The statements in the literature are somewhat conflicting about the effects of pregnancy. *Varaldo* (1905) and *Anufrejew* (1910) found an enlarged spleen with some hyperplastic Malphigian corpuscles in pregnant guinea-pigs. *Barcroft & Stevens*

(1928 a, b) and *Sleeth et al.* (1939), on the contrary, found that during pregnancy the exteriorized spleen became smaller during the last days of the gestation period.

It is of interest to note that a relation between the gonads and spleen has also been demonstrated clinically. *Naegeli* (1928) and *Gavazzeni* (1934) reported cases of splenomegaly associated with hypogenitalism, which returned to normal after splenectomy. *Radosavljevic et al.* (1929) suggested the spleen has an inhibitory effect on ovarian function. *Sauerbruch et al.* (1937) were of the opinion that the anterior pituitary lobe is of decisive importance in this effect, and that the secretion of gonadotropic hormones is inhibited by the spleen. Similar results were obtained by *Sakharoff* (1930) who found a premature development of the gonads in infantile experimental animals after splenectomy.

To sum up, the literature contains few data on the extent to which the different parts of the spleen contributes to variations in the size of the organ. The present study was undertaken to throw some light on this problem. For practical reasons it was limited to a determination of the quantitative relations between lymphatic and non-lymphatic splenic tissue in ovariectomized animals, pregnant animals, and female animals treated with oestrogens. In order to estimate the »reactive state« of the white pulp, the relative amount of pale centres in the lymphatic nodules, (here called *reaction centres*) was determined.

Terminology.

The following terms are used in the present paper to describe the various components of the lymphatic tissue of the spleen:

Reaction centres denote the central, pale parts in the fully differentiated lymphatic nodules (Fig. 1).

Other lymphatic tissue denotes the periarterial lymphatic tissue sheaths, the lymphatic nodules without reaction centres,

and the lymphatic tissue immediately surrounding the reaction centres.

Non-lymphatic tissue denotes the red pulp, the capsule, the trabeculae, and the vessel walls.

MATERIAL AND METHODS

Eighty adult, unmated, female guinea-pigs varying in age between 210 and 265 days were used. All the animals belonged to the same strain. They were kept in cages of the same size with one animal in each cage. Their food consisted of dry hay, swedes and wheat bran mixed with water. Water was given *ad libitum*. All animals were examined and weighed at regular intervals. Special attention was paid to the possible occurrence of scratches and signs of infection. All animals that showed signs of cutaneous infection, poor physical condition or changes in weight without demonstrable cause were excluded from the experiment. The skin and the viscera were carefully examined at post-mortem. If any abnormal conditions were then revealed, the animal was excluded from the experiment.

The animals were divided into four groups of 20 animals each. Care was then taken to ensure that the weight distribution in the different groups was as even as possible.

Group I: Animals gonadectomized 4 weeks before the start of the experiment. 7 animals were excluded.

Group II: Animals given subcutaneous injections of 10,000 I. U. of oestradiol monobenzoate every three days. Altogether 10 injections were given to each animal. 7 animals were excluded.

Group III: Animals mated with male guinea-pigs 10 days before the beginning of the experiment. 9 animals were excluded.

Group IV: Control animals, of which 6 were excluded.

*) The hormone preparation (Follidrin-benzoat-forte) were kindly placed at my disposal by Messrs. Astra, Söderfälje, Sweden.

Most of the animals excluded were discarded on account of infected cutaneous excoriations.

The experiments were started simultaneously on all the groups and went on for 32 days. The animals were weighed at the beginning of the experiment, and on every day thereafter, as well as at autopsy. The pregnant animals were also weighed at autopsy, after removal of the foetus and placenta (reduced weight).

All animals were killed by bleeding under ether anaesthesia. The spleen was removed immediately and weighed while still fresh. A transverse section, consisting of the middle third of the spleen, was excised, weighed and fixed at once in a mixture of equal parts of a saturated water solution of mercuric chloride and a 20 per cent solution of formalin. The material was embedded in paraffin, cut in 10 μ serial sections, and stained with haematoxylin and eosin. Special care was taken to ensure that all the material received the same histological treatment.

Using *Hammar's* (1931) method every fifth section was drawn on special paper of controlled uniform thickness with a projection apparatus with a magnification of 17 times. The contours of the transverse section, the border of the white pulp against the non-lymphatic tissue, and the contours of the reaction centres present were drawn. No differentiation was made between the dark border zone of the reaction centres, and the periarterial lymphatic tissue sheaths. The trabeculae, the capsule and the vessels were not shown (Fig. 1). The drawings were cut out, and the paper reproductions of the reaction centres, the remaining lymphatic tissue, and the non-lymphatic tissue were weighed separately to nearest 0.5 mg. With the help of the weights thus obtained, the amount of white pulp in the entire splenic tissue, and the amount of reaction centres, in the white pulp, were calculated and expressed as percentages of the total weight. An approximate calculation of these proportions in relation to the bodyweights was also made.

RESULTS

The various weights are given in the following tables.

Table 1 gives the mean figures for the body weights (BW) at autopsy, the increase in weight during the experiment, the weight of the fresh spleen (SW) and the ratio SW/BW calculated in gm. of spleen per kg. of body weight («relative spleen weight»). The body weight of the pregnant animals is given after removal of the contents of the uterus.

Table 1.
Relation between body weight and weight of spleen.

Group	No. of animals	Mean figure for			
		Body weight (BW) gm.	Increase in weight gm.	Spleen, fresh weight (SW) gm.	Ratio SW/BW gm /kg.
Control animals	14	513.2	62.2	0.733	1.42
Oestradiol-treated animals	13	504.6	58.3	0.970	1.92
Pregnant animals	11	516.3	73.5	0.867	1.67
Gonadectomized animals	13	582.3	157.3	0.630	1.08

It is seen from Table 1 that the gonadectomized animals show a considerable gain in body weight as compared with that of the other groups. The spleens of the oestradiol-treated animals show a somewhat larger fresh weight than that of the pregnant animals, and hence their SW/BW-ratios («relative spleen weight») were raised. This was in contrast to the low corresponding values of the gonadectomized animals.

Table 2 gives the mean figures for the amount of the white pulp (WP) expressed as a percentage of the amount of the whole spleen (S), and the proportion of reaction centres (RC) in the white pulp, also expressed as a percentage.

Table 2.

Relation between amount of the whole spleen, the white pulp and the reaction centres.

Group	No. of animals	Mean figure for		
		Ratio SW/BW gm./kg.	Ratio WP/S per cent	Ratio RC/WP per cent
Control animals	14	1.42	13.9	11.7
Oestradiol-treated animals	13	1.92	10.2	10.6
Pregnant animals	11	1.67	16.0	12.0
Gonadectomized animals	13	1.08	20.5	16.9

The proportion of white pulp in the gonadectomized and pregnant animals is thus higher than in the controls, whereas the oestradioltreated animals show rather lower figures. The statistical significance of these figures is shown in Table 3. The amount of reaction centres in the white pulp is practically identical in the first three groups, but a marked increase is found in the gonadectomized animals.

Table 3.

Differences between the various groups of guinea-pigs as regards the proportion of white pulp and reaction centres respectively.

Difference between	White pulp		Reaction centres	
	t ¹	p ²	t ¹	p ²
Controls and pregnant	6.24	<0.001	0.52	0.6
Controls and gonadectomized	8.26	<0.001	4.66	<0.001
Controls and oestradiol-treated	7.51	<0.001	1.94	0.05
Pregnant and gonadectomized	4.58	<0.001	1.68	0.1

1) t = the quotient of the difference and its mean error.

2) p = refers to the probability of the difference occurring by chance (calculated according to R. A. Fischer). A »p« of less than 0.05 indicates a significant difference.

Table 3 shows the difference between the various groups as regards the proportion of white pulp and reaction centres respectively.

It is thus seen that the differences between the amount of white pulp in the controls and the experimental animals are

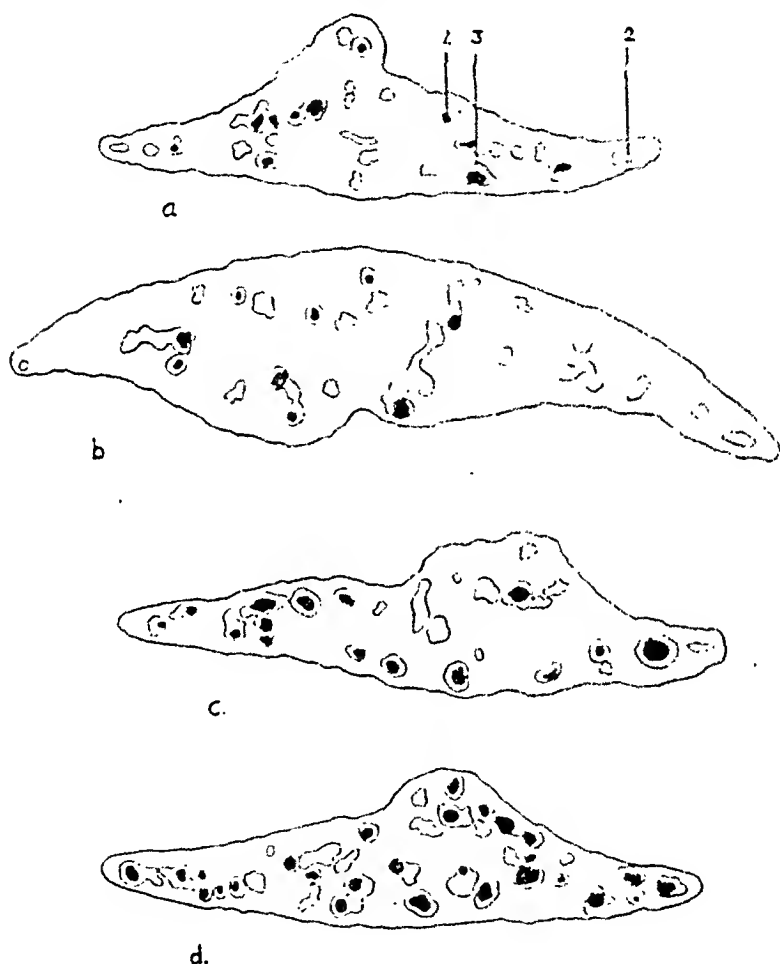


Fig. 1.

Contour drawings from the transverse sections of the spleen in the various experimental guinea-pigs, demonstrating the amount and condition of the white pulp in different types of spleen.

a. control animal; b. oestradiol-treated animal; c. pregnant animal; d. gonadectomized animal. 1. reaction centre (black); 2. undeveloped lymphatic nodule; 3. lymphocytic border zone surrounding the reaction centre. $\times 7.5$.

all statistically significant. As regards the proportion of reaction centres in the white pulp only in the gonadectomized animals is there a significant difference as compared with the controls.

Table 4 shows the amount of white pulp in relation to body weight.

Table 4.
The amount of white pulp in relation to body weight.

Group	No. of animals	Mean figure for	
		White pulp Whole spleen	Amount of white pulp per kg. body weight
Control animals	14	13.9	0.199
Oestradiol-treated animals	13	10.2	0.196
Pregnant animals	11	16.0	0.270
Gonadectomized animals	13	20.5	0.221

It is seen from the figures in the last column of Table 3 that the pregnant animals have the largest amount of white pulp in relation to their body weight. The gonadectomized animals also show an increase of the lymphatic tissue of the spleen but there is practically no difference between the amounts of lymphatic tissue in the spleens of the oestradiol treated and control animals.

Considering the reaction centres these appeared histologically to be of the same type in all the groups containing but little of nuclear debris, some mitotic figures and surrounded by a typical outer darkstaining zone of small lymphocytes.

DISCUSSION

The significance of the hormonal factors is difficult to interpret on the basis of the results obtained. The experiments

do not necessarily indicate that the action of the female gonads have a *direct influence* on the amount of the lymphatic tissue of the spleen but they evidently show that the various groups of animals have *different types of spleens* schematically shown in Fig. 1. According to v. Herrath (1939) there are two different types of spleens among mammals: the so-called »Abwehrtyp« with a well developed white pulp and opposed to that the »Speicherungstyp« i. e. with moderately developed lymphatic tissue.

It seems reasonable to interpret the differences in the types of spleen found in the different groups as related to the variations in the ability of the white pulp to respond to mild infectious or toxic irritants. The reactive ability of the white pulp seems to be modified by the female gonads.

The striking difference between the amount of white pulp in the various groups of animals is due to some extent to differences in the amount of non-lymphatic splenic tissue (see Table 4). The considerable increase in the amount of white pulp in the pregnant and gonadectomized animals as compared with the controls is, however, quite clear. The findings in the pregnant animals are in agreement with those described by Valardo (1905) and Anufrejew (1910). In the present investigation the spleens were examined in *the middle third of the pregnancy*, and this may explain why these organs were so large in contradiction to the small spleens found during the last part of pregnancy by Sleeth *et al.* (1939).

The comparatively small amount of white pulp in the spleens of the oestradioltreated animals may demonstrate the effect of very large amounts of oestradiol (100 000 I. U.). According to Selye *et al.* (1936) oestrogens given in sufficiently high doses are capable of producing an *alarm reaction* with involution of the white pulp. The action of the oestrogen given in this experiment may thus be considered as nonspecific.

It is seen from the figures in Table 2 that there is a positive correlation between the amount of reaction centres and the remaining lymphatic tissue of the spleen. (Correlation coefficient = $+ 0.71 \pm 0.07$). This implies that an increase in

the white pulp usually causes a proportionate increase in the amount of lymphatic nodules with developed reaction centres. The gonadectomized animals are an exception to this rule since they show a disproportionately large amount of reaction centres.

SUMMARY

The effect of gonadectomy, pregnancy and administration of oestradiol monobenzoate on the lymphatic tissue of the spleen is studied in 51 guinea-pigs.

In comparison with the controls, the spleens of the gonadectomized animals show a considerably increase in the amount of lymphatic tissue, and a disproportionately large amount of reaction centres.

The spleens of the pregnant animals also had a large amount of white pulp with a proportionally large amount of reaction centres.

The spleens of the oestradiol-treated animals were strikingly enlarged, with a relatively small amount of lymphatic tissue and a proportionately low content of reaction centres.

The differences between the amounts of white pulp, expressed as percentages of the whole spleen, were all statistically significant.

As regards the amount of reaction centres expressed as a percentage of the white pulp, only the gonadectomized animals showed a significant difference from the controls.

The reactive ability of the white splenic pulp appears to be influenced by the endocrine condition of the female gonads.

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VARIATIONS IN THE 24-HOURS' RHYTHM OF THE HEPATIC GLYCOGEN IN THE RABBIT FOLLOWING HYPOPHYSECTOMY

BY

JAN STAHLÉ

I. THE HEPATIC RHYTHM.

Since *Claude Bernard's* discovery of hepatic glycogen in 1856, much has been written concerning the rôle of the liver in carbohydrate metabolism. When *Forsgren* (1927) demonstrated that there was a special 24-hour rhythmic variation in the storage and production of hepatic glycogen, a special method of investigating liver function became available. In later publications, in which he also studied the formation of bile, the same worker found that there was an inverse relation between the formation of glycogen and of bile, as these processes compete with each other to some extent. Thus, when the liver shows a maximum storage of glycogen, the formation of bile is at a minimum and *vice versa*.

The 24-hour cycle in the function of the liver was subsequently studied in rats and mice by *Ågren, Wilander & Jorpes* (1931) and by *Holmgren* (1931). *Higgins, Berkson & Flock* (1932), however, were of the opinion, that the content of gly-

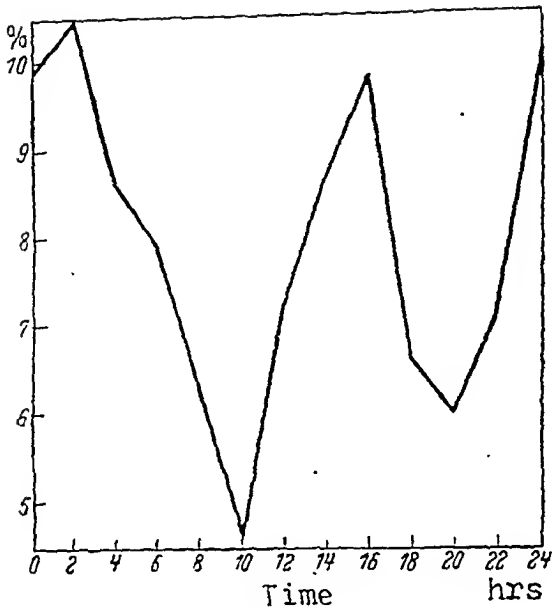


Fig. 1.

The hepatic glycogen content as described by Forsgren.

cogen, water, fat and protein in the liver, is, to a great extent determined, by the time needed for the intake of food, and that there is »no cyclic activity« in the liver itself. They did not, however, exclude the possibility that there is a primary gastro-intestinal rhythm which controls the effects seen, secondarily, in the liver.

Later workers (*von Euler & Holmquist*, 1934), were able to confirm the findings of Forsgren on a large number of rabbits, and *Möllerström* (1930) demonstrated a daily rhythmic excretion of urine dextrose, which is to a great extent independent of the intake of food. He also showed that in diabetics there was a daily cyclic variation in the blood sugar, in which the alimentary factors appear to be of minor importance. The investigations of *Gerritzen* (1940) on the excretion of substances in the urine in man, give further support to the theory of a hepatic rhythm.

The times in the diurnal cycle at which the maximum and minimum glycogen contents of the liver occur, vary in different species. *Sjögren*, *Nordenskjöld*, *Holmgren* & *Möller-*

ström (1938) investigated *inter alia* the glycogen variations in the rabbit. They demonstrated two glycogen maxima in the female rabbit, i. e. at 5 and 16 o'clock and two minima, i. e. a definite one at 9 o'clock and one less sharply defined between 22 and 3 o'clock. A similar cycle also occurs in male rabbits. The authors emphasize, however, that only twelve males were used in these experiments, and that the results may not be significant.

It is further demonstrated in the same paper that male rabbits have a larger capacity for glycogen storage than females. *Duel jr., Gulick, Grunewald & Cutler* (1934) showed that the amount of hepatic glycogen level found in the female is approximately 60 per cent of that of the male, and also that the glycogen capacity of the liver varies according to the age of the animal. *Duel jr., Butts, Hallman, Murray & Blunden*

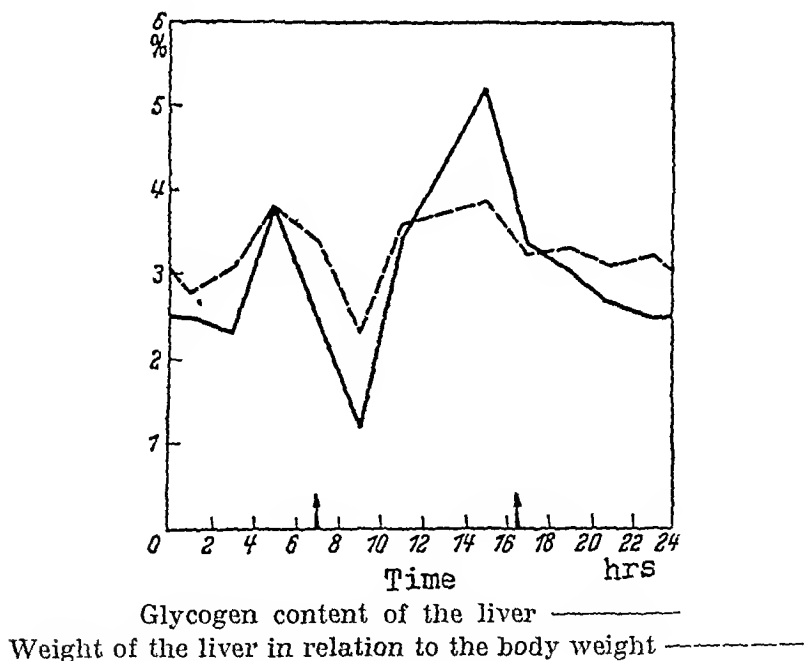


Fig. 2.

Female rabbits after feeding. The glycogen content in the liver, and the weight of the liver in relation to the body weight. (The arrows indicate the feeding time). According to *Sjögren, Nordenskjöld, Holmgren & Möllerström*,

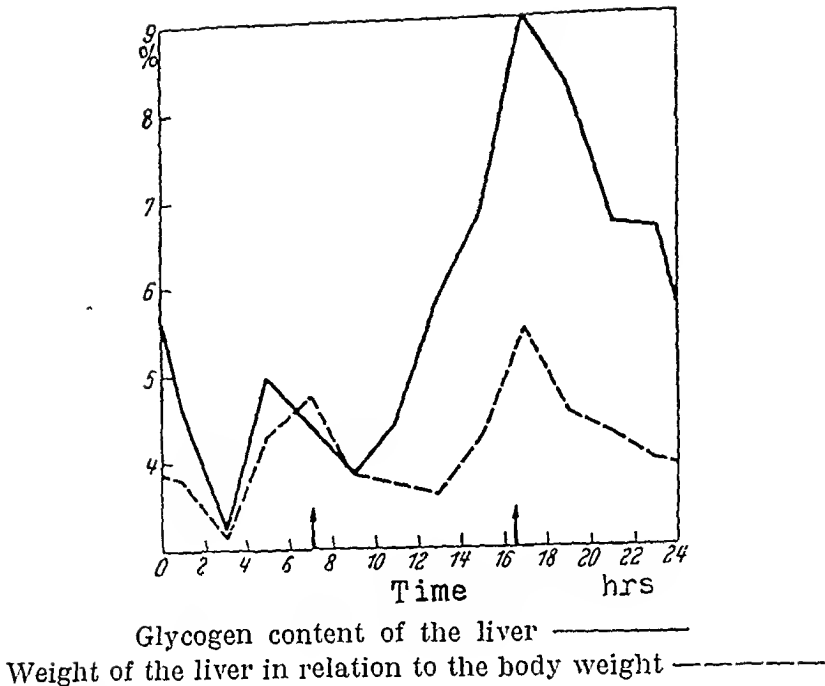


Fig. 3.

Male rabbits after feeding. Glycogen content in the liver, and weight of the liver in relation to the body weight. (The arrows indicate the feeding time). According to *Sjögren, Nordenskjöld, Holmgren & Möllerström*.

(1937) demonstrated that in mice and rabbits the glycogen content of the liver is greatest during the period of sexual maturity and lower in young and old animals.

A number of organs in the body show similar 24-hour rhythmical variations. The monograph by Jores (1937), on the subject should be consulted.

II. THE REGULATING FACTOR.

The existence of an endogenous 24-hour' rhythm in the liver glycogen metabolism appears to be fairly well established. The problem then arises: what is the regulating factor? Some workers believe that, it is the adrenals, others that the pituitary body is responsible.

That the adrenals play an important rôle in the regulating

mechanism is shown by the investigations of *von Euler & Holmquist* (1934), who demonstrated that the concentration of adrenaline both in the adrenals and in the blood, undergoes daily cyclic variations with a maximum value corresponding approximately to the minimum content value of the hepatic glycogen, and vice versa. Moreover, *Ågren* (1935) found in adrenalectomized rabbits, that the glycogen content of the liver was low at all times of the day, and that a 24-hours' rhythm could not be demonstrated. He considered that cortin was probably the regulating factor. *Bergman & Klein* (1943) excised the adrenal glands of rats and then administered an extract of the adrenal cortex. By this means they were able to restore a normal balance between the content of glycogen and the weight of the liver.

The effect of the pituitary on the hepatic rhythm in rats was studied by *Jores* (1940) who found that after hypophysectomy, the glycogen and bile pigment rhythm was considerably disturbed, but not altogether inhibited. The glycogen maximum during the night, demonstrated by *Forsgren* and others still occurred. It is also of some interest in this connection to note that *van Nieuwenhizen* (1941) demonstrated in a number of cases in man disturbances in the carbohydrate absorption associated with various degrees of pituitary insufficiency.

The problem of the effect of the adrenals and the pituitary is complicated by the question of the interrelationship between these organs. Thus *Houssay, Biasotti & Mazzacco* (1933), found that the weight of the adrenals was reduced by 38 per cent after destruction of the pituitary body in dogs by means of electric cauterization, and *Houssay & Mazzacco* (1933) demonstrated that the amount of adrenaline per unit weight of adrenal gland decreased in hypophysectomized dogs. *Ingle* (1942) showed that if the anterior lobe of pituitary is removed, the adrenal cortex undergoes considerable atrophy. According to *Long* (1939), if hypophysectomized rabbits are treated with an extract of the adrenal cortex, then the decreased hepatic glycogen level caused by the atrophy of the

adrenal cortex is once more raised. *Houssay* (1942) summarises the relation of the anterior lobe of the pituitary gland and of the adrenals, to the carbohydrate metabolism, and has demonstrated that the amount of hepatic glycogen is not affected by hypophysectomy, provided that the animal is given food *ad libitum* post-operatively. In fasting animals, on the other hand, the glycogen values are extremely low. *Houssay* does not, however, state at what time of the day the glycogen determination was made, nor does he discuss the problem from the aspect of the hepatic glycogen rhythm.

In studies on experimental diabetes in the rabbit, *Iversen* (1947) found that the hepatic glycogen was not significantly different from the normal in adrenalectomy, in destruction of the adrenal medulla, or in adrenalectomy combined with pre-treatment with an extract of the adrenal cortex. In hypophysectomized animals, however, it was somewhat less than in normal animals. It is difficult to assess the value of these findings, since *Iversen* did not take into account the fact that the time at which the determinations are made effects the figures obtained, both for the amount of blood sugar and of hepatic glycogen.

To sum up, it can be said that the rôle of the pituitary in the regulation of the 24-hour cyclic variations of hepatic glycogen is as yet not fully known. The aim of the present investigation is to throw some light on this problem. Its primary object is to determine the weight of the liver as a percentage of the body weight, after hypophysectomy, and also to measure glycogen content as a percentage of the weight of the liver at two fixed times during the day, when a glycogen minimum and maximum respectively occur in normal animals.

III. METHOD.

Female rabbits were used as experimental animals. Hypophysectomy was performed, by the method of *Jacobssohn & Westman* (1940), on twenty rabbits, and twenty were used as controls. Numal

(10 per cent allylisopropyl barbituric acid, Hoffman-La Roche, Bâle) was used as an anæsthetic. Since hypophysectomy causes considerable damage to the organism, postoperative complications are not unusual. Penicillin was therefore administered to several animals in order to prevent postoperative pneumonia. In addition, the days just after the operation, it was necessary to inject glucose intravenously to some animals to overcome imminent hypoplycaemia.

Both groups of animals were given food *ad libitum*. This consisted of hay, crushed oats, and swedes. Fourteen days after hypophysectomy the animals were sacrificed, the liver was excised and weighed and the glycogen content determined. Twenty animals (10 experimental animals and 10 controls) were killed at 10 o'clock, when a glycogen minimum is believed to occur, and a corresponding number at 17 o'clock, i. e. approximately at the time of the glycogen maximum. The determination of the hepatic glycogen was performed by *Bertrand's* method. In each case one gram of liver from the same lobe was examined. Two determinations were made for each animal, and the mean figure obtained was taken as the basis for the investigation. The glycogen was dissolved in 60 per cent potassium hydroxide solution, washed carefully in alcohol and ether, and hydrolyzed in 5 per cent hydrochloric acid, after which the reducing sugar was determined volumetrically.

The pituitary fossa was sectioned in all animals in order to make sure that the hypophysectomy had been complete, and this was found to be the case in all but two animals, in which small pieces of the pituitary were found on microscopic examination. Since it can be assumed that any function of the pituitary gland in these cases was very considerably decreased, these figures were also included in the investigation.

Histological sections were prepared from all the livers and stained both with carmine and by Mallory's method with Forsgren's modification. Since the findings were similar to those described by Forsgren, and since no apparent difference between the liver structure in the hypophysectomized and the normal animals could be observed, no description of the appearance of the sections will be given here.

IV. RESULTS OF THE INVESTIGATIONS.

The times for sacrificing the animals were chosen to coincide with those of the maximum and minimum hepatic glycogen since it was at such times that any variations between

the glycogen content of the experimental and control animals could be expected to be most obvious, should the pituitary gland have any regulating effect on the hepatic glycogen.

Table 1 shows the figures obtained at 10 and 17 o'clock, when a glycogen maximum and minimum respectively can be considered to occur.

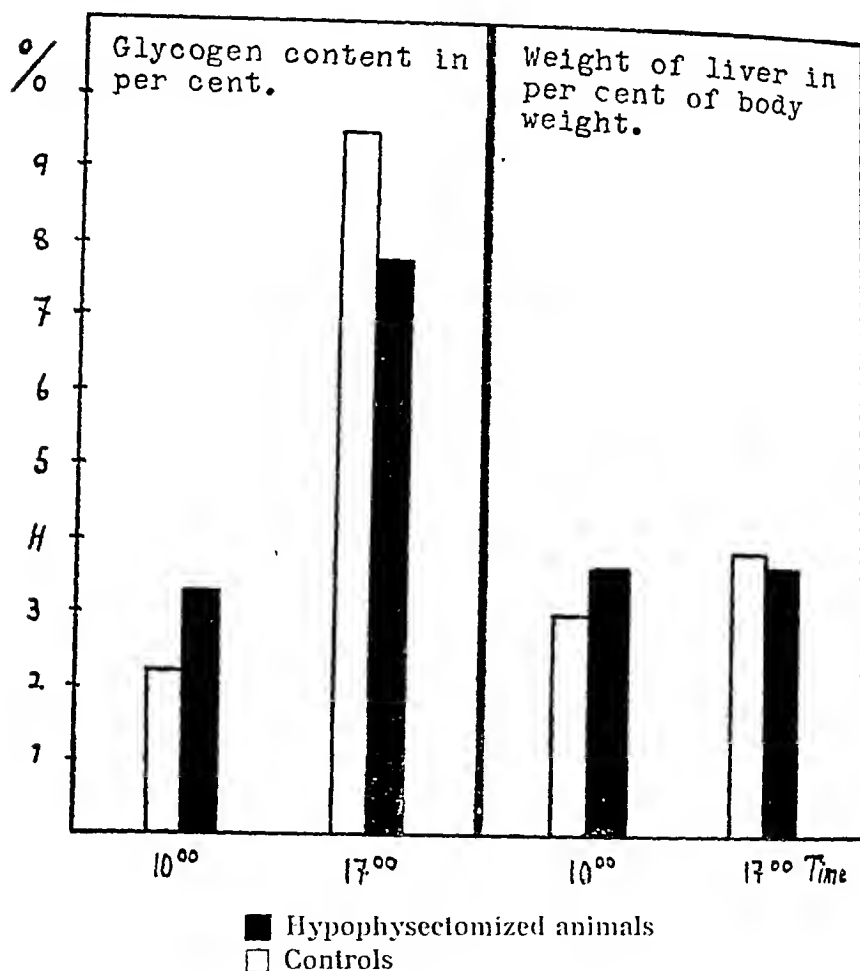
Table 1.

Liver weight and glycogen content in hypophysectomized animals and in controls.

	Time of analysis	Weight of liver as a percentage of body weight	Glycogen content in per cent
Hypophysectomized animals	10.00 hrs	3.55 ± 0.20	3.20 ± 0.20
Controls	10.00 hrs	2.91 ± 0.14	2.13 ± 0.17
Difference		0.64 ± 0.24	1.07 ± 0.26
Hypophysectomized animals	17.00 hrs	3.64 ± 0.13	7.67 ± 0.31
Controls	17.00 hrs	3.79 ± 0.18	9.44 ± 0.54
Difference		0.15 ± 0.22	1.77 ± 0.62

It is seen from the table that the weight of the liver of the hypophysectomized animals, expressed as a percentage of the body weight, shows a probable increase at 10 o'clock (difference: 0.64 ± 0.24). Moreover, the hepatic glycogen content, as a percentage of the liver weight at the same time, shows a statistically significant increase (difference: 1.07 ± 0.26) in comparison with that of the controls. At 17 o'clock, however, the conditions are to some extent reversed. The weight of the liver as a percentage of the body weight shows no statistically significant variation, whereas the glycogen content of the controls as a percentage shows an increase as compared with the experimental animals (difference: 1.77 ± 0.62).

The results of the experiments can be expressed graphically as follows:



It is seen from this diagram that the experimental animals show much smaller glycogen variations than the controls. The glycogen minimum and maximum are, however, still present. The pituitary evidently has some regulating effect on the 24-hours' rhythm of the glycogen metabolism, but hypophysectomy does not cause a cessation of the hepatic rhythm. Either the hepatic rhythm is only partly regulated by the pituitary, or some other organ acts as a temporary regulator. There is, of course, a third possibility, i. e. that the pituitary is not in

any way connected with the 24-hour cyclic variations and that the decreased glycogen value observed at the period of the expected glycogen maximum are due to a traumatic effect of the operation. But this does not explain the fact that the glycogen figures for the experimental animals at 10 o'clock show a statistically significant increase over those of the controls and also that the postoperative loss of body weight is usually slight and that several animals even showed a small increase in weight.

Since the percentage figures for the glycogen content in the experimental animals at 10 o'clock are considerably higher than in the controls and lower at 17 o'clock, it is likely that the pituitary has some regulating effect on the glycogen metabolism.

SUMMARY.

An investigation was carried out on 40 female rabbits in order to ascertain the possible influence of hypophysectomy on the 24-hour cyclic variations of the hepatic glycogen. The animals were divided into two groups, of which the first was killed at 10 o'clock and the second at 17 o'clock. Each group consisted of 10 experimental animals and 10 controls. Food was given *ad libitum*. The glycogen content was determined as a percentage of the weight of the liver, and the weight of the liver as a percentage of the body weight.

At 10 o'clock, when the controls showed a minimum glycogen content, the hypophysectomized animals showed a statistically significant increase in the glycogen content expressed as a percentage of the liver weight (difference: 1.07 ± 0.26). At 17 o'clock, however, when the controls showed a glycogen maximum, a decrease was found in the experimental animals which was almost significant (difference: 1.77 ± 0.62).

The relation between the weight of the liver and the body weight showed no statistically significant difference between the experimental and the control animals. At 10 o'clock, how-

ever, the experimental animals showed a probable increase in the weight of the liver (difference: 0.64 ± 0.24) whereas at 17 o'clock no difference in the weight of the liver of the two groups could be observed.

The daily rhythm in the hepatic glycogen metabolism thus showed smaller variations than in the controls, but was nevertheless apparent. This decrease in the variation was probably caused by the cessation of the hypophyseal function.

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THE ANTIGEN AND PYROGEN CONTENT IN
COMMERCIAL CHORIONIC GONADOTROPHIN
PREPARATIONS, WITH REFERENCE TO
THEIR CLINICAL APPLICATION

BY

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& M. TØNNESEN

Treatment with preparations of chorionic gonadotrophin often leads to certain toxic complications. In the worst of cases these well-known complications, consisting especially of pyrexia and its associated symptoms, require either that the treatment be suspended or that it be continued with such small doses that no hormonal effect can be expected. The cause of these unfortunate reactions is that the preparations contain impurities originally present in the urine of pregnant women.

The active principles of many drugs of biological origin have not yet been prepared in a pure state, at best they may be described as purified concentrates of the active principles. The past few years have seen the introduction of tests for impurities as well as for the determination of the potency of the drugs. This applies to substances such as heparin, liver and certain vitamin preparations, but no definite standard has yet been set up for the purity of chorionic gonadotrophin preparations.

Kjems & Pedersen-Bjergaard (1942) suggested using the antigen content in chorionic gonadotrophin preparations as a

measure of the degree of purity and introducing a specific limit for the content of antigens. The present work may be regarded as an amplification of that idea.

When making a biological evaluation of the impurities in a preparation, we have concentrated on investigating its content of antigens and pyrogens. Both groups of substances are found in urine and will be present in the chorionic gonadotrophin preparations, as they have a marked tendency to accompany the gonadotrophic activity through the processes both of purification and isolation. The antigens are chiefly of human origin, whereas the pyrogens are essentially products of bacterial growth in the urine. The toxic effects observed from treatment with chorionic gonadotrophin are presumably to a large extent due to the pyrogens present in the preparations. Antigens are not foreign to the human organism and anaphylactic symptoms or serum disease have not apparently been observed in patients treated with chorionic gonadotrophin. Thus, although antigens are in themselves practically innocuous, they will provide some indication about the purity of the preparations since they remain closely associated with the hormone and pyrogen in the process of purification.

Our purpose has been to establish, by means of comparative clinical and biological observations, a simple method for determining how much the amount of antigen and pyrogen present in a given preparation of chorionic gonadotrophin effects its clinical use.

TECHNIQUE

The investigation was performed as follows:

Twelve preparations of chorionic gonadotrophin of various degrees of purity were tested. The clinical evaluation was made by injecting 1500 I. U. intramuscularly into healthy women. We chose this dose because *Rydberg & Madsen* (1947) use doses of this order when treating amenorrhea and functional sterility. 2, 4 and 6 hours after the injection the women's temperatures were taken. In addition, they answered

questions as to their general condition, especially with regard to sensations of cold or shivering, nausea, vomiting, and local tenderness around the site of injection. 20—40 injections of each of the 12 preparations were given and altogether 100 women were injected. A total of 350 observations were made.

For the antigen test we used the classical anaphylaxis test on guinea-pigs. The principles of the quantitative titration of antigens were described by *Walzer & Grove* (1925). Our experimental technique, previously described by *Kjems & Pedersen-Bjergaard* (1942), was as follows: Young guinea-pigs weighing 250—300 gm. were sensitized with the preparation by means of three subcutaneous injections at intervals of five days. On the 25th day after the first sensitizing dose, the ice cooled shock dose was injected intravenously in the jugular vein. The shock dose used was 3.000 I. U. of a rather impure chorionic gonadotrophin preparation. If within three minutes of the injection this dose is capable of causing a fatal shock in the sensitized animal, the reaction is regarded as positive. We have determined the quantity of the preparation, expressed in international units, which, used as one of the sensitizing doses, is capable of causing fatal shock in 50 per cent of the animals. This quantity we have called AD_{50} . For the preliminary determination of the antigen content in a preparation we used three animals for each dose, and for the ultimate titration ten animals for each dose.

Measurement of the pyrogenic activity of preparations was made on rabbits weighing 1800—2200 gm. The quantity of substance to be tested was dissolved in 5 ml. physiological saline prepared with pyrogen-free water, and injected into an ear vein of a rabbit immediately after the rectal temperature was taken. The temperature was then taken after $1\frac{1}{2}$, 3 and 5 hours. The maximum temperature difference is then an expression of the quantity of pyrogen injected. We have determined the quantity, expressed in international units, which causes a rise of 0.7° C in the temperature. This value is called $PD_{0.7}$. Because of individual variations, determinations by this method are rather uncertain and hence, we used a minimum of 15 rabbits for each dose.

Table 1.

The antigen and pyrogen contents in chorionic gonadotrophin preparations of various purities.

1	2	3	4	5
No.	No. of clinical observations	Febrile reaction, in per cent	AD ₅₀	PD _{0.7}
1	33	0	22	3000
2	29	7	3	350
3	29	14	4.8	175
4	40	15	2.4	175
5	41	22	6.2	150
6	18	33	2.5	150
7	21	52	2.5	100
8	28	54	0.8	40
9	30	43	0.4	35
10	40	43	4.2	30
11	22	50	0.4	30
12	24	70	0.9	20

RESULTS

The results of the investigation will be seen in Table 1. In the first column the numbers of the preparations are given, in the second, the number of observations forming the basis of the clinical evaluation, while the third column shows the percentage of cases of fever observed among the patients. The last two columns show the relative content of anaphylaxis- and pyrexia-inducing substances as determined on guinea-pigs and rabbits respectively. The preparations are listed according to their content of pyrogens, as measured on rabbits, preparation No. 1 having the lowest pyrogenic activity per unit and No. 12 the highest.

As already stated, the rectal temperatures of the women were taken. The symptoms recorded by the women treated with preparations of chorionic gonadotrophin consisted of cold, shivering, nausea, local tenderness around the site of injection, and sometimes other discomforts. In many instances, however, these observations were of so subjective a nature

and their relation to the injection so uncertain that we did not consider it advisable to include them here. Nausea following injection was complained of in less than 10 per cent of all patients; and as it was consistently found with all the preparations used, no great significance can be attributed to it.

As regards local irritation, preparation No. 1 gave no cause for complaint, but with Nos. 2 to 6 about 50 per cent and with Nos. 7 to 12 about 80 per cent of the patients complained of pain at the site of injection. Although there was no close relation between the degree of purity of the preparations and their irritant properties, the three figures: 0, 50 and 80 per cent are all the same in the last two groups, in accordance with expectation.

Shivering was naturally observed in some febrile patients, usually when the rise in temperature was more than 1° C. As a general rule the increase was only about 0.5 to 1.0° C, but in some cases there were increases of from 1° to 2° C, and indeed in a few instances, when preparation No. 12 was employed, as much as 2.5° C. In view of the marked individual variability of the reactions of man to pyrogenic substances, we have determined the pyrogenic effect only qualitatively using as our criterion the occurrence of an abnormally high body temperature. On the other hand, when dealing with rabbits, which must be regarded as a much more homogenous material, we aimed at expressing the results quantitatively e. g. $PD_{0.7}$ is the dose in I. U. which within the limits variability, just causes a temperature rise of 0.7° C.

It will be seen from the table that the preparations have a $PD_{0.7}$ varying from 20 to 3,000; this means that preparation No. 1 is 150 times as pure as No. 12 from the pyrogenic point of view. This difference is reflected in Column 3, which shows that when preparation No. 12 is administered, a fever reaction may be expected in about three-quarters of the cases, whereas there will be no pyrexia when preparation No. 1 is used.

On the whole, no complications were observed from the use of No. 1. Preparations Nos. 2 to 11 have effects interme-

diate between that of pure and impure preparations, and it will be seen that there is a satisfactory agreement between the values in Cols. 3 and 5. The value AD_{50} in Col. 4 (the dose in I. U. which produces anaphylactic shock in five out of ten animals) varies from 22 to 0.4, a difference of about 1 to 50. Generally speaking there is close agreement between AD_{50} and $PD_{0.7}$: the purer preparations have an AD_{50} higher than 2.5, and the impure ones have an AD_{50} of less than 2.5. Preparation No. 10 is an exception; antigenically it is relatively pure, but pyrogenically very impure. Thus an antigen determination alone is not sufficient to determine the purity of a preparation.

If now we were to define the limits of purity from the results of our present investigations, we should say that as preparation No. 1 produces no complications and No. 2 hardly any, the permissible content of impurities, as expressed by the AD_{50} and the $PD_{0.7}$, must lie within the limits observed in these two preparations. In other words, the AD_{50} must lie between 3 and 22, and the $PD_{0.7}$ must lie between 350 and 3,000. *We would therefore suggest that a chorionic gonadotrophin preparation to be used clinically in the form and dosage described above should have an AD_{50} higher than 10, and a $PD_{0.7}$ higher than 1,000.*

Having worked out these standards, we next considered the question of how closely they are attained by the best known commercial preparations at present available. In all, we tested ten preparations from well-known chemical firms in six different countries. The results will be seen in Table 2, in which the preparations are arranged according to the pyrogen content in the same way as in Table 1. Only preparations Nos. 1, 2 and 3 came up to the suggested standard; the remainder were found to be very toxic. Preparations Nos. 5 and 6 contain only a small quantity of antigen as compared with their pyrogen content, and No. 5 shows that a preparation may very well comply with the antigen test and still be highly pyrogenic. Thus even if the contents of these two impurities run parallel in most cases, there are exceptions, pro-

Table 2.

The antigen and pyrogen contents in commercial chorionic gonadotrophin preparations.

Preparation	AD ₅₀	PD _{0.7}
I	22	3000
II	—	2000
III	25	1500
IV	2	75
V	25	40
VI	6	15
VII	1.5	15
VIII	2	15
IX	3	10
X	0.5	5

bably because some factories have processes with which large quantities of proteins are removed leaving the polysaccharides of which the pyrogens presumably consist, still associated with the hormone. The injections of 1500 I. U. of preparations Nos. 4 to 10 are certain to induce considerable temperature reactions. However, the low doses, usually from 50 to 500 I. U., recommended in some of the literature accompanying the preparations, would seem to indicate that it is more often the impurity of the preparation rather than its therapeutic potency, which determines the dosage suggested. The observation shows the need for introducing definite standards of purity for chorionic gonadotrophin preparations.

SUMMARY

A number of chorionic gonadotrophin preparations of various degrees of purity were tested for antigenic and pyrogenic substances. These tests were accompanied by an investigation of the extent to which these preparations cause toxic complications when used clinically. It was found that there is such a close correlation between the degree of purity of a prepara-

tion and its clinical value, that the usefulness of a preparation can be inferred from a determination of the antigen and pyrogen contents, and especially of the latter. On the basis of these experiments the authors suggest that preparations should be tested for their purity. Such a test was performed on ten different products from ten different factories. Only three preparations out of the ten were found to be satisfactory.

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THE FORMATION OF ANTIHORMONES IN WOMEN TREATED WITH PREGNANT MARES' SERUM HORMONE (ANTEX).*)

BY

ERLING ØSTERGAARD and CHRISTIAN HAMBURGER

The formation of antigonadotrophic substances in patients under treatment with gonadotrophic hormones has been demonstrated by *Rowlands & Spence* (1939), *Jailer & Leathem* (1940), *Østergaard* (1942), *Leathem & Abarbanel* (1943), *Leathem* (1944), *Leathem & Rakoff* (1948 a, b), and *Hamburger* (1948).

It appears from our present knowledge of the mechanism involved in the formation of antihormones that this process is of an immunological nature and that only gonadotrophins originating from a species other than that of the recipient can induce in the recipient the formation of antihormone against the particular gonadotrophin.

Accordingly, the gonadotrophins used by above mentioned investigators in man have in all cases been of an origin other than human, i. e. pregnant mares' serum or sheep hypophyses. In spite of numerous investigations it has never been possible to demonstrate in man antihormones resulting from the ad-

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ministration of chorionic or other gonadotrophins of human origin. It is not necessary to accept as an exception to this rule the fact that *Segaloff & Parson* (1947) found that the serum of a patient treated with chorionic gonadotrophin inactivated this substance; for the patient had been treated with hypophyseal gonadotrophin of foreign origin before the treatment with chorionic gonadotrophin was started, and this presumably was the cause of the formation of antihormone in their case.

It should be mentioned that, under certain conditions, prolonged treatment with gonadotrophins may result in the formation in the receptor animal of substances which are not antigonadotrophic but progonadotrophic, i. e. they augment the activity of the particular gonadotrophin when injected with it into test animals. (*Collip*, 1937, *Thompson*, 1937, *Rowlands*, 1938, *Katzmann et al.*, 1939, and *Østergaard*, 1942). In the animal experiments of *Collip*, *Thompson* and *Rowlands* continued treatment with gonadotrophin resulted in a preliminary period of progonadotrophic activity of the receptor animals' serum, which after some time changed its activity from progonadotrophic into antigonadotrophic. According to the explanation of this phenomenon given by *Thompson* and *Rowlands*, it is reasonable in such cases to look upon the progonadotrophic activity as the first step in the process of formation of antihormones.

Progonadotrophic activity in the blood of man treated with gonadotrophic hormones has not yet been recorded. But it appears from the experimental data in the paper by *Leathem & Rakoff* (1948 a) that at least 3 of the blood specimens from their patients showed a progonadotrophic activity; the authors, however, take no notice of this fact.

In *Østergaard's* thesis (1942) it was shown that continued and prolonged treatment of man with Antex (pregnant mares' serum hormone) quite frequently leads to the formation of a specific antihormone in the recipient, whereas an intense treatment of short duration (3000 I. U. of Antex daily in 5 consecutive days) did not result in the formation of antihor-

mone in 10 cases investigated, of which 4 received a repetition of the short treatment a month later. It was therefore suggested that the best method of giving Antex in cases of ovarian insufficiency consisted in such short periods of treatment to be repeated later if necessary.

In 1948, however, *Hamburger* demonstrated the presence of antihormone in a patient after 3 such brief Antex-treatments, and this led the authors to investigate the question: How often in a greater number of cases does the repetition of such short periods of treatment with Antex lead to the formation of antihormone?

PATIENTS AND TREATMENT

The patients who were examined for antihormone were all women treated for different degrees of ovarian insufficiency, in the gynecological department of the Frederiksberg Hospital, Copenhagen.

Up to the present 20 cases have been investigated with exactly the same technique as is described below. We have not found it necessary for our purpose to give all the case histories in detail. The reader is referred to Table 1, which gives the case number, age of the patient, diagnosis of the case including our opinion as to the degree of ovarian insufficiency, details of the treatment, results of treatment with regard to restoration of menstrual cycle, details of the investigation for antihormone and an evaluation of the different blood specimens.

The patients were all in-patients for some time and underwent the usual investigations for such cases, including endometrial biopsy, hormone analyses, basal metabolism and X-ray examination of the sella turcica. The assessment of the degree of ovarian insufficiency is based upon the clinical condition of the patient, extreme insufficiency being reserved for cases with increased excretion of gonadotrophin or very pro-

nounced atrophy of the genital organs. Accordingly the cases may be grouped as follows:

Primary amenorrhea, extreme insufficiency, 4 cases;

Secondary amenorrhea, 9 cases;

Oligomenorrhea, extreme insufficiency, 1 case;

Oligomenorrhea, 5 cases;

Meno-metrorrhagia, 1 case.

Three of the patients complained of infertility.

The treatment has been very uniform. All patients have received a series of 5 injections of pregnant mares' serum preparations (Antex Leo) given on 5 consecutive days, followed by 3 injections of chorionic hormone preparations (Physex Leo) given every other day. The doses of Antex have been 1500 I. U. or 3000 I. U. each day and the doses of Physex 1500 I. U. per injection. This Antex-Physex treatment has been repeated once, twice or three times, with an interval of one to several months if the effect on the ovary of a single treatment was not found sufficient to induce menstruation.

Because of the established fact that chorionic hormone preparations do not give rise to the formation of antihormone in man the Physex-treatment has not been recorded in Table 1. But, as pointed out in the footnote of the table, it has to be remembered when assessing the results of the treatment, that all patients received Physex as well as Antex.

TECHNIQUE IN INVESTIGATION FOR ANTIHORMONE

The authors have pointed out in a recent paper, (*Hamburger & Østergaard, 1949*) that it is not possible to give an exact value or titer for the antigonadotrophic activity of a serum, because the titer found depends on the relative proportion of gonadotrophin to antigonadotrophin in the serum.

But under strictly standardized experimental conditions, using the same amount of serum to the same amount of gonadotrophin as we have done throughout this investigation, it

is quite possible to obtain a rough estimate of the antigonadotrophic activity of the different specimens of serum.

Hence the assay for antihormone has been carried out in the following manner: On the 21st day after the last injection of Antex, a specimen of blood about 50 ml. is drawn from a cubital vein. After spontaneous coagulation of the blood, the serum is pipetted off. The serum and a solution of a standardized Antex-preparation (No. 460910) were in all cases mixed in the proportion of 1 ml. serum to 10 I. U. of Antex.

Groups of 5—12 (usually 8) immature female mice are injected subcutaneously with 2 or 3 different doses of this mixture. The animals are killed about 96 hours after the beginning of the experiment and the dose-response curves obtained for the average ovarian weight are compared with the corresponding curves obtained in mice treated with the Antex-preparation alone. The gonadotrophic activity of the mixture is calculated in this way and expressed as I. U. of Antex.

If the gonadotrophic activity of the mixture is found to be about 10 I. U. per ml., (from 7—13 I. U.), the serum has had no anti- or progonadotrophic activity and has been considered »neutral«. If the gonadotrophic activity has been less than 7 I. U. or more than 13 I. U. per ml., the serum specimen has been considered »antigonadotrophic«, or »progonadotrophic«, respectively.

As test animals we have used immature female mice in this investigation, because at the beginning of the investigation we had many mice at our disposal and only a few rats. Our experimental protocols have shown, however, that the variation in weight of ovaries is greater in mice than in our strain of rats. Using immature female rats as test animals it should be possible, therefore, to decrease the number of test animals per dose of serum and thus diminish the amount of serum necessary to carry out an investigation for antihormone.

RESULTS

Table 1 gives the results of the treatment and of the investigation for antihormone.

Table 1.

Results of treatment and investigation for antihormone in 20 cases of ovarian insufficiency.

Case No.	Age	Diagnosis	Treatment. (Amount of Antex I. U.)*)	Menstrual bleeding after treatment**)	Investigation for antihormone.		
					Gonadotrophic activity of 1 ml. serum + 10 I. U. Antex		Evaluation of blood specimen
					Date	I. U.	
1	32	Oligomen- orrhea. Secondary amenorrhea of 1 year's duration.	1) Oct. 46 3000 I. U. × 5	0	15/11-46	14	progon.
			2) Dec. 46 3000 I. U. × 5	+	8/1-47	3	antigon.
					17/4-47	8	neutral.
			3) Febr. 48 3000 I. U. × 5	++	18/3-48	0	antigon.
2	25	Primary amenorrhea. Extreme insuf- ficiency.	1) 1940 3000 I. U. × 5	+			
			2) Oct. 46 3000 I. U. × 5	0	7/11-46	11	neutral.
			3) Dec. 46 3000 I. U. × 5	0	29/12-46	8	neutral.

*) Each series of 5 consecutive injections of Antex has been followed by 3 injections of 1500 I. U. of Physex given every other day.

**) 0 means no menstrual bleeding in response to treatment.
+ means one menstrual bleeding in response to treatment.
++ means several menstrual bleedings observed after treatment, in some cases with an interval of 2—3 months.

Case No.	Age	Diagnosis	Treatment. (Amount of Antex I. U.)*	Menstrual bleeding after treatment**)	Investigation for antihormone.		
					Gonadotrophic activity of 1 ml. serum + 10 I. U. Antex		Evaluation of blood specimen
					Date	I. U.	
3	24	Secondary amenorrhea of 9 months' duration.	1) Febr. 48 1500 I. U. × 5	+			
			2) March 48 1500 I. U. × 5	0			
			3) April 48 3000 I. U. × 5	++	29/6-48 21/8-48 16/11-48	0 0 10	antigon. antigon. neutral.
4	24	Oligomen- orrhea. Now sec. amenorrhea of 15 months' duration.	1) Sept. 46 1500 I. U. × 5	+			
			2) Febr. 47 1500 I. U. × 5	+	15/3-47	14	progon.
			3) July 47 1500 I. U. × 5	0 No report later	18/8-47	10	neutral.
5	21	Oligomen- orrhea. Now sec. amenorrhea of 1 year's duration. Infertile marriage.	1) Aug. 46 1500 I. U. × 5	+			
			2) Dec. 47 1500 I. U. × 5	0	29/12-47	17	progon.
			3) Jan. 48 3000 I. U. × 5	++	30/1-48	12	neutral.

Case No.	Age	Diagnosis	Treatment. (Amount of Antex I. U.)*	Menstrual bleeding after treatment*)	Investigation for antihormone.		
					Gonadotrophic activity of 1 ml. serum + 10 I. U. Antex		Evaluation of blood specimen
					Date	I. U.	
6	38	Secondary amenorrhea of 6 months' duration.	1) April 48 1500 I. U. × 5	0			
			2) May 48 3000 I. U. × 5	+	4/6-48	9	neutral.
			3) June 48 1500 I. U. × 5	++	3/7-48	0	antigon.
7	20	Primary amenorrhea. Hypometa- bolism. Extreme insuf- ficiency.	1) April 48 1500 I. U. × 5	0	18/5-48	16	progon.
			2) May 48 1500 I. U. × 5	0	19/6-48	10	neutral.
			3) Aug. 48 3000 I. U. × 5	0	17/9-48	0	antigon.
					21/11-48	10	neutral.
8	16	meno-me- trorrhagia.	1) Dec. 47 1500 I. U. × 5	+			
			2) April 48 1500 I. U. × 5	+			
			3) June 48 1500 I. U. × 5	++	21/7-48	0	antigon.

Case No.	Age	Diagnosis	Treatment. (Amount of Antex I. U.)*	Menstrual bleeding after treatment**)	Investigation for antihormone.		
					Gonadotrophic activity of 1 ml. serum + 10 I. U. Antex		Evaluation of blood specimen
					Date	I. U.	
9	20	Oligomen- orrhea. 9-16 months' interval. Extreme insuf- ficiency.	1) Febr. 46 3000 I. U. × 5	+			
			2) March 46 3000 I. U. × 5	+			
			3) May 46 3000 I. U. × 5	0	14/6-46	3	antigon.
			4) Sept. 46 1500 I. U. × 5	0	5/10-46	2	antigon.
10	26	Oligomen- orrhea. Infertile marriage.	1) 31/7-4/8 48 1500 I. U. × 5	+	25/8-48	16	progon.
			2) 27/8-31/8 48 1500 I. U. × 5	0	21/9-48 21/10-48	15 18	progon. progon.
			3) 21/10-25/10 48 1500 I. U. × 5	+	8/11-48 15/11-48 16/12-48	10 6 20	neutral. antigon. progon.
				under observ- ation			
11	20	Oligomen- orrhea.	1) Oct. 46 1500 I. U. × 5	+	9/11-46	13	neutral.
			2) Dec. 46 1500 I. U. × 5	++	10/1-47	8	neutral.

Case No.	Age	Diagnosis	Treatment. (Amount of Antex I. U.)*)	Menstrual bleeding after treatment**)	Investigation for antihormone.		
					Gonadotrophic activity of 1 ml. serum + 10 I. U. Antex		Evaluation of blood specimen
					Date	I. U.	
12	20	Secondary amenorrhea of 1 year's duration.	1) Nov. 46 1500 I. U. × 5	+	19/12-46	3	antigon.
			2) Dec. 46 1500 I. U. × 5	+	18/1-47	0	antigon.
			3) Jan. 47 1500 I. U. × 5	0			
13	22	Primary amenorrhea. Hypometab- olism. Extreme insuf- ficiency.	1) March 47 3000 I. U. × 5	(+)	5/4-47	12	neutral.
			2) Sept. 47 3000 I. U. × 5	0	4/10-47	0	antigon.
14	24	Oligomen- orrhea.	1) April 48 1500 I. U. × 5	0			
			2) May 48 1500 I. U. × 5	++	10/6-48	13	neutral.
15	26	Secondary amenorrhea of 6 months' duration. Infertile.	1) Sept. 47 1500 I. U. × 5	+	6/10-47	15	progon.
			2) Oct. 47 1500 I. U. × 5	0 Preg- nant***)	3/11-47	10	neutral. (conception about 15/10)

***) 16/6 48 parturition, twins, otherwise normal delivery. The normal infants, a boy and a girl weighed 2800 gm. and 2600 gm. and were thought to be about 3 weeks premature.

Case No.	Age	Diagnosis	Treatment. (Amount of Antex I. U.)*	Menstrual bleeding after treatment**	Investigation for antihormone.		
					Gonadotrophic activity of 1 ml. serum + 10 I. U. Antex		Evaluation of blood specimen.
					Date	I. U.	
16	21	Secondary amenorrhea of 15 months' duration.	1) Nov. 47 1500 I. U. × 5 2) Dec. 47 1500 I. U. × 5	+			
				++	9/1-48	2	antigon.
17	19	Oligomen- orrhea.	1) July 48 1500 I. U. × 5 2) Aug. 48 1500 I. U. × 5	+	14/8-48	10	neutral.
				++	10/9-48	16	progon.
					9/10-48	10	neutral.
18	20	Primary amenorrhea. Hypometab- olism. Extreme insuf- ficiency.	1) Aug. 48 1500 I. U. × 5 2) Sept. 48 3000 I. U. × 5	0	27/8-48	9	neutral.
				0	24/9-48	0	antigon.
					6/10-48	0	antigon.
					22/2-49	13	neutral.
19	27	Secondary amenorrhea of 6 months' duration.	1) 29/9-3/10 48 3000 I. U. × 5 2) Dec. 48 1500 I. U. × 5	+	25/10-48	15	progon.
				0	5/1-49	0	antigon.
				Under observ- ation			
20	19	Oligomen- orrhea.	1) Nov. 46 1500 I. U. × 5	++	19/12-46	12	neutral.

Case 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, were treated with 3 courses of Antex+Physex; case 9 received 4 such courses of treatment. As a result of the treatment case 1, 3, 5, 6, 8, menstruated and had their menstrual cycles restored.

Case 11, 13, 14, 15, 16, 17, 18, 19 were treated with 2 courses of Antex+Physex. Case 11, 14, 15, 16, 17, benefitted by the treatment and had their menstrual cycles restored. Case 16 became pregnant as a result of the treatment. Case 20 had her menstrual cycle restored after one treatment with Antex +Physex.

In all, 10 of the 20 patients benefitted from the treatment.

A total of 51 treatments with Antex+Physex resulted in 31 menstrual bleedings occurring at the usual and expected time, 2—3 weeks after the last injection of Antex.

A total of 48 specimens of blood have been investigated, 37 of which are given in Table 2. Eleven of the blood examinations have been undertaken *after 1 course of Antex-treatment*. Five of these specimens showed a progonadotrophic, 5 a neutral and 1 an antigonadotrophic activity.

Sixteen blood specimens were examined *after 2 courses of Antex-treatment*. Three of these specimens were progonadotrophic, 7 neutral and 6 antigonadotrophic.

Ten specimens were examined *after 3 courses of Antex-treatment*. None of these specimens were progonadotrophic, 3 were neutral, and 7 were antigonadotrophic.

Hence the formation of antihormone has been observed in 1 out of 11 cases after one treatment, in 6 out of 16 after 2 treatments and in 7 out of 10 cases after 3 treatments. It is thus obvious that an increasing number of Antex-treatments increases the probability of the treatment resulting in the formation of antihormone.

On the other hand, the presence of progonadotrophic sera is most often found after the first treatment, a few times after the second treatment and not at all after the third treatment. It appears that in some cases a period of progonadotrophic activity of the serum is usually a preliminary to the later de-

velopment of antigonadotrophic activity. Cases 1, 7, 10, 19, illustrate this fact.

The question as to how long after its first appearance antihormone can still be found in the blood has been answered in cases 1, 3, 7, 10, 18, in which it was found to have disappeared from the blood in 3 — 5 — 2 — 1 and 5 months respectively.

Table 2.

Survey of results of investigation for antihormone in 20 cases.

Number of Antex-treatments before the blood specimen is drawn.	Number of blood specimens showing the following activity:			Total number of blood specimens.
	progonadotrophic	neutral	antigonadotrophic	
1	5	5	1	11
2	3	7	6	16
3	0	3	7	10

DISCUSSION

It is obvious from this investigation that even short courses of Antex-treatment may result in the formation of antihormone, particularly if such short treatments have been repeated. In his former investigations Østergaard (1942) was unable to find antihormone in patients treated in this way, but this may be due to the fact that he only investigated 10 cases after one short period of treatment, and 4 after two courses of treatments, and that the investigation for antihormone was undertaken too early, namely 2 weeks after the last injection of Antex as against 3 weeks after the last injection in the present work; this, possibly, together with the small number of cases investigated after two courses of treatments may explain the earlier negative findings.

From the present work it appears that one short Antex-

treatment can be given almost without any risk of antihormone formation, but even after 2 short periods of Antex-treatments the probability of finding antihormone in the blood of patients is about 33 per cent and that with increasing numbers of courses of treatment the incidence of antihormone formation increases considerably. Any injection of Antex given at a time when the blood contains antihormone against Antex must be regarded as almost useless, besides which it will further increase the formation of antihormone.

Table 1 gives some information on this point. It is possible that the failure of the treatment No. 3 and 4 in case 9 and of treatment No. 3 in case 12 is due to the presence of antihormone at the time of treatment. In case 19 it is not unlikely that the progonadotrophic activity found in the blood in October had changed to antigonadotrophic in December, when the second and unsuccessful treatment was given.

On the other hand case 1, 3, 6, 8, 16 illustrate that a second or third treatment may give rise to the formation of antihormone at a later period and yet result in menstrual bleeding occurring at a time when the blood contains antihormone, showing that this antihormone has not interfered with the occurrence of later spontaneous menstruations in these cases. This is in agreement with the findings of *Rowlands & Spence* (1939) and *Østergaard* (1942), who showed that the specific antihormone against Antex did not inhibit human hypophyseal or chorionic gonadotrophin. Neither did it inhibit hypophyseal gonadotrophin from other sources.

The treatment of ovarian insufficiency is not the main subject of this work. Yet, a brief comment on our results of treatment seems to be appropriate. With a rate of cure of 50 per cent we are in agreement with the earlier published results of *Rydberg* (1939), *Rydberg & Østergaard* (1939), *Rydberg & Pedersen-Bjergaard* (1943), who used this same type of treatment. Our doses of Antex have in many cases been only half those used by the above-mentioned authors; we have found it more suitable to use this lower dose in young patients with presumably mild ovarian insufficiency. In our

patients the lower dosage has been at least as effective as the double dosage, which has been used in some of the cases. But this naturally depends on the type of case; 5 of our cases were classified as extreme insufficiency in which not even the large doses were able to bring about a menstrual cycle. It is questionable whether gonadotrophic hormone is an adequate remedy in such cases of ovarian insufficiency in which the receptor organ, i. e. the ovary has obviously lost or never has had any ability to react to gonadotrophins.

Our investigations does not allow of any definite statement as to the ratio of dosage to incidence of formation of antihormone. The fact, however, that antihormone was formed in 75 per cent of the patients treated with the large doses as against 50 per cent of the patients treated with smaller doses, suggests that the dosage may be of importance in this respect.

To get a fair opinion of the value of gonadotrophic hormones in the treatment of ovarian insufficiency it is important to choose only such cases as are suited to this form of treatment and to consider the complication resulting from the formation of antihormone. Furthermore the Antex-treatment ought not to be given at a time when antihormone is already present in the blood.

Treatment with gonadotrophic hormone is especially indicated in infertility due to ovarian insufficiency. Three of our cases were of this category; one of them became pregnant as a result of the treatment. *Rydberg & Madsen* (1947) have published several similar cases.

The practical consequences and conclusions of the present work can be stated as follows:

If a case of ovarian insufficiency is found suitable for this form of treatment with Antex-Physex, a single course of treatment will often suffice to produce a cure.

Should a second treatment be necessary this can be given without much risk that formation of antihormone will interfere with the effect of this treatment.

In cases where further treatment is desirable the blood ought to be examined for the presence of antihormone against

Antex, and when present, the treatment should be postponed until the antihormone has been eliminated from the blood. Or, one could choose another gonadotrophin for further treatment, e. g. chorionic gonadotrophin or a combination of this hormone with hypophyseal extract (e. g. »Synapoidin«).

SUMMARY

Twenty women complaining of different degrees of ovarian insufficiency received a combined treatment with pregnant mares' serum gonadotrophin (Antex, Leo) and chorionic gonadotrophin (Physex, Leo).

The treatment consisted of daily injections of 1500 I. U. (or 3000 I. U.) of Antex for 5 days followed by 3 injections of 1500 I. U. of Physex given every other day. If further stimulation of the ovaries was found advisable, the Antex+Physex-treatment was repeated once or twice after intervals of from one to several months.

Fifty-one such treatments resulted in 31 menstrual bleedings. Ten of the 20 patients continued to menstruate spontaneously after cessation of the treatment.

In all the 20 patients the blood was examined once or several times for the presence of antihormone against Antex. Usually this assay was performed on the 21st day after the last Antex injection.

Antihormone against Antex was found in the blood in 1 out of 11 cases after one course of treatment, in 6 out of 16 cases after two courses of treatment and in 7 out of 10 cases after three courses of treatment.

The antihormone disappeared from the blood in from 1 to 5 months. In 4 of the cases the blood showed a progonadotrophic activity after the first or second treatment and an antigonadotrophic activity after later treatments.

The practical consequences of these investigations for the treatment of man with pregnant mares' serum gonadotrophin are discussed.

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THE THIOURACIL DERIVATIVES AND THEIR USE*)

BY

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We know from experience that new therapeutic methods often at first give rise to unfounded enthusiasm. Indications for their application are given too widely, until experience leads to a more cautious view. This has also been found to apply to the subject of the present paper, i. e. the thiouracil derivatives and their use.

Thiouracil and its derivatives are the most important of the goiterproducing substances, whose ability to suppress the secretory activity of the thyroid can be used in practice in the treatment of thyrotoxicosis.

Before the introduction of thiouracil into clinical use at the beginning of the present decade, iodine was the only drug which could influence the course of thyrotoxicosis. It is known, however, that not all cases of thyrotoxicosis react favourably enough to iodine, to make it possible to operate safely. This is especially true of those cases in which thyrotoxicosis has developed following the administration of iodine or where the condition has deteriorated during a course of iodine therapy. Compared with iodine therapy, however, thiouracil has the

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advantage that in practically every case it eliminates all the symptoms of thyrotoxicosis with the exception of the eye symptoms. It is thus possible to change thyrotoxicosis into euthyreosis, and, if the dosage is sufficiently high and the period of treatment long enough, into hypothyreosis and myxoedema. The effect becomes apparent more slowly than with iodine, but is more stable and lasts for as long as the treatment is continued. There is thus less difficulty in the preoperative treatment of cases with severe heart failure which require prolonged treatment with cardiac drugs in order to prepare the patient for the operation. The same applies too to other severe forms of the illness. In Finland the reaction to iodine in cases of thyrotoxicosis is as a rule rapid and satisfactory, allowing the operation to be carried out safely. It is, as a rule, preferable to avoid thiouracil therapy when it is not absolutely necessary. Hence iodine should be used in the preoperative therapy of all mild and moderate cases, while thiouracil should be reserved for severe and complicated cases.

Cases which have previously been treated with iodine react more slowly to thiouracil. The same is true of thyrotoxicoses with nodular goitre, which is the common type of the disease in this country. However, in both of these cases the full effect is obtained if the treatment is continued long enough.

Thiouracil suppresses the thyroid activity and finally eliminates thyrotoxicosis by preventing the utilisation of iodine for the synthesis of the thyroid hormone. With the suppression of the thyroid activity, the thyrotrophic effect of the adenohypophysis will increase. This causes an increase of the hyperplasia in the thyroid and also of the hyperemia, which may be sufficient to cause serious technical difficulties in the operation. Since thiouracil therapy does not prevent the usual effect of iodine, i. e. reduction of hyperplasia and hyperemia in the thyroid, this disadvantage is eliminated, to some extent at least, by the simultaneous administration of iodine. American investigators have lately started using iodine in addition to thiouracil during the whole of the preoperative

period of treatment, and not merely towards the end, as was formerly the case (*Bartels*, 1948). I have recently used this method myself. The thiouracil medication is stopped on the day of operation, the iodine therapy being continued until the postoperative reaction subsides, when it should be immediately discontinued in order to avoid the development of hypothyreosis.

The danger that thiouracil therapy might cause enlargement of the thyroid is less in the relatively short preoperative treatment than in a more prolonged period of medication, and it is also smaller when the dosage is kept relatively low. I have encountered this complication only in one case in which the patient had been treated with thiouracil for a long time and finally operation became necessary on account of pressure symptom and thyrotoxicosis. In cases of low cervical or intrathoracic goitres, however, increased pressure symptoms may occur even in the preoperative treatment.

Another complication, which ought to be mentioned, is exophthalmos and other »thyrotoxic« eye symptoms, associated with prolonged thiouracil medication. It is known that the eye symptoms differ from all other thyrotoxic symptoms in their ability to appear and disappear quite independently of the other features of the disease. According to current views, they are probably due to the action of thyrotrophin secretion or to some other humoral action of the adenohypophysis. It was mentioned earlier that increase of the pituitary thyrotrophin action plays an important role in the effects produced by thiouracil. In my opinion this could explain the relatively frequent occurrence of exophthalmos etc. in connection with prolonged thiouracil therapy.

The chief disadvantage, however, is the undoubted toxicity of thiouracil which in some cases gives rise to undesirable complications such as »drug fever«, headache, vomiting and diarrhoea, various skin affections like urticaria, scleroderma and exfoliative dermatitis, enlarged lymph glands in the neck and swollen salivary glands, conjunctivitis, arthropathic edema in the lower extremities, neuritis and psychoses, and

indications of kidney and liver injury. Even more important, however, is the fact that thiouracil, as well as the derivatives so far synthesised, have marked toxic effects on the bone marrow which, in sensitive patients, may cause granulocytopenia and even agranulocytosis, thereby endangering the patient's life. Anyone who, like myself, has seen an acute agranulocytosis develop during careful clinical observation, is not likely to forget this danger. That frequently repeated examinations of the blood may not eliminate this danger, is illustrated by my case, in which the blood picture was quite normal on the day before the onset of agranulocytosis. The risk, however, becomes less when thiouracil medication is begun clinically, and continued as outpatient with lower doses, but experience has nevertheless shown, that agranulocytosis may develop at any stage of thiouracil therapy and independently of the dosage. The patient can of course be warned to discontinue the medication at once, if he observes symptoms such as a rise of body temperature, sore throat and mouth, or swollen lymph glands in the neck. However, this requires a certain amount of intelligence and observation on the part of the patient, and ideal co-operation with the physician, which can rarely be depended on. Thiouracil therapy, without a subsequent operation, may be successful, since it has been shown that the hyperplasia of the thyroid epithelium has a tendency to become involuted during the treatment (*Frisk, 1947*) and the therapy can be stopped without a relapse of the disease. In this respect thiouracil medication differs from iodine therapy without subsequent operation. The latter is successful in only a few exceptional cases and usually leads to various complications. On the other hand, thiouracil therapy must be continued for months or even years, and must therefore even if only for economic reasons, be largely carried out as outpatients. For reasons already explained, outpatient thiouracil medication involves in my opinion such grave dangers for the patient that I consider it is contra-indicated in all but exceptional cases. It is evidently much more dangerous than prolonged outpatient medication with amidopyrine, and it might well be

asked whether any conscientious physician would take this responsibility. As will be described later, my experience has shown that hypersensitivity to thiouracil probably is greater in Finland than elsewhere, and this too calls for caution. In the latest reports from the USA there is a definite tendency for increased caution in the treatment of thyrotoxicosis with thiouracil derivatives alone, without subsequent operation (*Bartels*, 1948, *Berlin et al.*, 1948, *Lockwood*, 1948). This change of opinion is partly due to the comparatively large number of deaths from agranulocytosis, of which *Cope*, has reported 27. Another reason is that distinct histological and biological signs of malignant degenerative changes in the thyroid epithelium in connection with prolonged administration of thiouracil have lately been observed not only in animal experiments (*Money & Rawson*, 1948) but also in man (*Money & Rawson*, 1948, *Purves & Griesbach*, 1947, *Berlin et al.*, 1948, a. o.)

Ever since it became apparent that administration of thiouracil and its derivatives involves the risk of agranulocytosis and thus endangers the life of the patient, attempts have been made to synthesise a derivative possessing a maximum anti-thyrotoxic effect combined with a minimum injurious effect on the bone-marrow. Of the derivatives so far tested, methyl- and 6-propylthiouracil have been found to satisfy these requirements best. Both of these drugs are now obtainable in Finland (Methylthiouracil, Medica; Meturasil, Orion; Propylthiouracil, Medica). Opinions have differed as to which of these two compounds is to be preferred, but it seems that the question is now settled in favour of propylthiouracil, especially since *Bartels* (1948), one of the foremost authorities in this field, having extensive material available at the Lahey-Clinic, concluded that the frequency of toxic reactions was 10 per cent with methylthiouracil, as compared with only 2 per cent with propylthiouracil. In a paper presented to the Congress of Internal Medicine in Copenhagen last summer, I came to a similar conclusion on the basis of my own material. Since then I have had further experience which supports this.

view, as will be seen below. That even propylthiouracil may cause granulocytopenia and agranulocytosis is now certain (Crile, 1947, Bartels, 1948). The material described below shows that there are not only cases in which methylthiouracil has a deleterious effect on the blood, and in which propylthiouracil is well tolerated, but also cases in which the reverse is true, propylthiouracil being injurious and methylthiouracil harmless. Thus, I have observed two cases of granulocytopenia, which developed during methylthiouracil therapy while propylthiouracil was harmless and conversely one case of hemorrhagic diathesis following propylthiouracil treatment while methylthiouracil was safe. For the time being, we may conclude that propylthiouracil is definitely to be preferred though methylthiouracil should also be available.

Williams et al. (1948) have quite recently published their first results with iodised thiouracil, a preparation which seems to combine the thorough effect of thiouracil with the rapid action of iodine on the thyrotoxicosis syndrome. The results appear to be better than those obtained with these two substances, used either separately or together.

At present we must make up our minds about two questions: which of the two derivatives, methyl- or propylthiouracil, should be used and what are the indications for their use with particular reference to the local conditions in Finland?

I have tried to answer these questions in earlier papers (*Wahlberg*, 1946, 1947, 1948), some of which are still unpublished, on the basis of my own material. This material is still not very extensive but should together with other workers' reports and generally acknowledged facts lead to some conclusions on the type of treatment to be followed.

MATERIAL

My material consists of 65 cases, all of which I have examined personally. In 55 of these cases thiouracil therapy was given, or its use continued, on my advice. The remaining 10

cases had been treated earlier by other physicians. Some of these cases came to me after the thiouracil medication had been stopped, while the others were sent to me whilst under treatment.

The longest period of observation is only two years and two months. Four cases are at present receiving treatment. Methyl- as well as propylthiouracil treatment was used in 5 cases.

The maximum dose of methylthiouracil has usually been 0.4 gm. or of propylthiouracil 0.2 gm. per day, administered in several doses during the day. During the last six months, however, the daily dose of propylthiouracil has in most cases been 0.3 gm.

4 cases were treated as outpatients only while in 5 cases the treatment was continued as outpatients with a lower dosage, after it had been begun as inpatients. All these cases were treated at a time when I was not yet convinced of the dangers of ambulatory therapy. It may be mentioned here that in 5 out of these 9 cases complications occurred during the outpatient treatment, so that in 3 cases the treatment had to be discontinued and in 2 cases the preparation changed.

Ten cases have been treated preoperatively. Of these, 7 were very severe cases of thyrotoxicosis with severe chronic heart failure, including two cases of auricular fibrillation, one with bundle branch block, one with advanced bronchial asthma and one with a very recently healed, bilateral pulmonary tuberculosis; in 4 out of these 7 cases thyrotoxicosis had developed during iodine therapy. As regards the remaining 3 cases, thiouracil medication had been started in two cases by another physician and continued after I had been consulted; in my opinion, there was no indication in either of these cases for a preoperative thiouracil medication. In the last case of this group, the patient had been discharged from the hospital by another physician after two months treatment, and instructed to continue the treatment as outpatient, which was done for several months without any control. When I was consulted the patient had a large goitre with pressure symp-

toms which had developed during the treatment and which, together with persistent thyrotoxicosis, were indications for operation.

In all cases treated preoperatively the result was favourable. In one of the patients who had auricular fibrillation which persisted after the operation, a fatal embolism of the brain occurred after a satisfactory convalescence which was entirely free from complications. This patient had for several years suffered from severe heart failure and auricular fibrillation. During the preoperative treatment the heart was completely compensated and all clinical symptoms disappeared except the fibrillation. I can see no reason to assume that there was any causal connection between the embolus and the preoperative treatment or the operation.

Complications were seen in 15 cases out of 65, i. e. in nearly 25 per cent, which is an unusually high proportion.

During the *methylothiouracil* treatment complications occurred in 11 cases out of 24, i. e. in almost 50 per cent of the cases. These included one case of acute agranulocytosis, which developed during inpatient treatment in spite of careful observation. The patient recovered with penicillin administration. There were also two cases of granulocytopenia which subsided when treatment was continued with propylthiouracil. Still another case deserves to be especially mentioned. After treatment for one day with methylothiouracil, massive, soft edema developed in the patient's legs. The treatment was discontinued and the edema disappeared. Another trial with methylothiouracil was made a week later, and edema promptly reappeared, to disappear again immediately treatment was stopped. It did not appear again when treatment was continued with propylthiouracil. After treatment was completed, the patient became symptom-free and has had no relapse in the ensuing year.

On the other hand, out of 46 patients treated with *propylthiouracil*, only 4, i. e. less than one-tenth, had complications. These included one case of hemorrhagic diathesis with prolonged coagulation time and lowered prothrombin index; all

symptoms subsided when the treatment was continued with methylthiouracil. One case developed colitis after two days' treatment with propylthiouracil. The patient became symptom-free when the therapy was discontinued but with propylthiouracil immediately became ill again. The medication was then definitely stopped, but the colitis persisted for a month with fairly grave symptoms and with changes in the colon which could be demonstrated by X-rays.

I have mentioned in earlier papers (Wahlberg, 1946, 1947, 1948) that I have tried to shorten the period of thiouracil treatment and, at the same time, to intensify the effect and avoid outpatient treatment, by a combination of inpatient therapy with thiouracil and X-rays for a period of one month. This series now consists of 37 cases of which 4 are still under treatment. The patients first received thiouracil for a few days and, when the symptoms of thyrotoxicosis had subsided, X-ray therapy was begun. This has consisted of 8 radiations of altogether 1.100 r. The X-ray treatment has been tolerated particularly well, and without a single marked reaction. The thiouracil therapy has been continued throughout the stay in hospital for about 4 weeks. At the beginning, an attempt was made to continue the treatment as outpatients with lower doses but it had to be abandoned on account of complications in 3 successive cases. Hence this treatment was stopped when the patient was discharged from hospital and has been used only on inpatients for almost two years. The patients have been examined 2 to 3 months later and, when necessary, have received one or two courses of X-ray therapy either in hospital or as outpatients, and with or without thiouracil depending on circumstances. Also in these cases thiouracil has not been used in outpatients for the last two years. Such complementary X-ray treatment has hitherto been necessary in 6 cases out of the 37. In one case one course was used, in two cases two and in one case three courses; all these cases were treated in the period: autumn 1946—winter 1947, when the trials with a combined thiouracil-X-ray therapy were begun. In order to increase the effect and to reduce the need for such comple-

mentary X-ray therapy the dosage of X-rays has recently been increased to 10 exposures of altogether 1800 r.

The results of the inpatient treatment with thiouracil and X-rays are encouraging. Of the 37 cases treated, 23 became completely symptom-free and 5 showed definite improvement, while the result was uncertain in 2 cases, and unsatisfactory in 3. Four cases are undergoing treatment at present.

Although the material is small and the period of observation short. I feel, that the procedure I have proposed rests on sound premises. The thiouracil medication is at present exclusively reserved for inpatients which thus tends to reduce its disadvantages to a minimum. By giving the patients thiouracil first for a few days prior to the X-ray therapy, tolerance to this treatment is increased. It is even possible that the extreme hyperplasia in the thyroid epithelium, brought about by the thiouracil therapy, produces a favourable condition for the effect of X-ray. Finally we have here another example of the simultaneous application of two agents with a wholly dissimilar effect on the disease. Experience has shown that such procedures may have a favourable effect on the result.

My impression of the 37 cases treated up to the present is very favourable and in my opinion this procedure might well be recommended as a standard method for the treatment of thyrotoxicosis with thiouracil without a subsequent operation.

The indications must be strictly adhered to: operated cases with subsequent relapsing or persisting thyrotoxicosis, where there are no indications for a new operation, and unoperated mild cases in which goitre is absent, or small and diffuse, or contains only small, soft nodules.

Several trials have been made on the value of thiouracil therapy in cases of cardiac or coronary insufficiency in the absence of thyrotoxicosis. The idea has been to lighten the burden of the heart by reducing the thyroid activity. My material includes a case of chronic myocardial injury where a progressing heart failure caused difficulties in the treatment. The result of a 4 weeks' therapy with propylthiouracil and X-rays in the manner described above were completely satis-

factory: there have been no untoward symptoms of hypothyroidism, and the patient has remained fully compensated for over a year with a small dose of digitalis, which prior to the treatment was not sufficient.

There was also a case of severe coronary insufficiency, in which the patient became completely symptom-free. She had been operated on 8 years previously for thyrotoxicosis but there were no definite residual symptoms of this disease, nor was there any relapse of the goitre.

CONCLUSIONS

In conclusion, I wish to mention those indications which, in my opinion, should be followed for thiouracil therapy in Finland. These indications are based on my personal experience as well as on the work of other investigations and on generally acknowledged facts.

I. As preoperative treatment in

1. cases which do not react satisfactory to iodine.
2. cases which *a priori* seem unsuitable for iodine therapy, as for instance:
 - a. cases in which thyrotoxicosis has developed in connection with iodine treatment.
 - b. cases, where the course of the illness has taken an unfavourable turn during the iodine treatment.
 - c. cases of severe thyrotoxicosis with severe heart failure, in which the preoperative therapy must necessarily include prolonged treatment with cardiac drugs («thyrocardiacs»), and
 - d. other severe cases in which the prospects of preparing the patient for operation solely with iodine seem poor.

II. As independent therapy in

1. cases of recurrent or persistent thyrotoxicosis following a *lege artis* operation, where there are no indications for a new operation.
2. mild cases in which goitre is absent or small and diffuse, or contains only small soft nodules, and
3. possibly also in those extremely rare cases where operation cannot be performed for one reason or another.

SUMMARY

1. Cases of thyrotoxicosis with nodular goitre are generally not suited for treatment with thiouracil derivatives as the only therapy.
2. Thiouracil medication should be used only for inpatients with rare exceptions.
3. Inpatient thiouracil treatment, combined with a simultaneous X-ray therapy for about one month can be recommended, provided that the above indications are observed.
4. In preoperative thiouracil therapy, iodine should be administered simultaneously throughout the treatment. After the operation the thiouracil treatment is immediately stopped, and the iodine therapy stopped as soon as the postoperative reaction has subsided.
5. Propylthiouracil is definitely preferred, but methylthiouracil may be tried in those cases in which propylthiouracil therapy leads to complications.
6. Treatment with thiouracil derivatives should only be used when other methods fail.

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RENAL CRISES FOLLOWING THE EXTIRPATION OF PARATHYROID ADENOMATA

BY

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In this paper a case of generalised osteitis fibrosa is described. The patient was for a time treated with stilboestrol, apparently with some success, but ultimately died following the operative removal of a parathyroid adenoma, showing signs of tetany and uremia. The author discusses the possible effects of stilboestrol. A review is also given of those crises which may occur following the removal of parathyroid adenomata, and attempts are made to analyse the cause of these crises, and particularly of the oliguria and the changes associated with it.

The case was typical of generalised osteitis fibrosa with skeletal changes, raised blood calcium, lowered blood phosphorus, polyuria and increased excretion of calcium and phosphorus in the urine. The patient was operated on twice without definite parathyroid adenoma being found and without improvement in her condition. It was therefore considered justifiable to attempt to help the patient by trying another line of treatment.

In cases of hyperparathyroidism there is either an adenoma in one or more of the parathyroid glands or a diffuse hyperplasia of several glands (*Castleman & Mallory, 1935, 1937*). Patients with several adenomata or hyperplasia of several

glands are unusual. *Cope* (1941) found hyperplasia in 10 per cent of a series of 60 cases submitted to operation. The occurrence of changes in several of the glands has given rise to discussion as to whether the change in the parathyroids are caused by an alteration in some factor which has a controlling action over the parathyroid glands.

Mellgren (1943, 1945), *Mellgren et al.* (1946) and *Wilton* (1945, 1946) have observed characteristic histological changes in the anterior lobe of the hypophysis in cases of hyperparathyroidism and have thus given substance to the suggestion that hyperparathyroidism may be caused by a change in the hypophysis. It has been thought that an increased amount of parathyrotrophic hormone might be formed in the anterior lobe of the hypophysis in such cases. *Wilton* considers that certain cases of generalised osteitis fibrosa are due to overfunction of the hypophysis. This question will not be discussed here, but the author is of the opinion (*Törnblom*, 1947) that it is not known whether the hypophysis produces a parathyrotrophic hormone.

As is well known, a marked effect is often observed when metastasizing carcinoma of the prostate is treated with synthetic oestrogens (*Kahle et al.*, 1942, *Watkinson et al.*, 1944). This is due to the fact that the prostatic epithelium, even when it has been the site of malignant degeneration, is affected by the testosterone produced in the testes (*Huggins et al.*, 1941, *Sullivan et al.*, 1942). The production of gonadotrophic hormone by the hypophysis is inhibited by the administration of such substances as stilboestrol and this leads to a decreased production of testosterone and secondarily to atrophic changes in the prostatic tissues. It is possible that oestrogen inhibits the production by the anterior lobe not only of gonadotrophic hormones, but also of other hormones, and hence it was thought that administration of stilboestrol might inhibit the overproduction of parathyrotrophic hormone if such was present. A trial of stilboestrol therapy was considered justifiable on these grounds.

DESCRIPTION OF THE CASE

The patient was an unmarried woman aged 35 and a professional cook. She was admitted to the surgical department in February 1945. She had met with an accident while tobogganing and had fractures of the olecranon and of the neck of the femur. She had previously been fairly healthy. For the three months previous to the accident she had been troubled with pain in both knees. During the last month she had become tired, slept a great deal and had pain in the small of the back. These complaints had forced her to give up her work as a cook a fortnight before her admission to hospital. She had suffered from such pronounced muscular weakness that she had to push herself up with her hands when she wanted to get up from a chair. Her appetite had become poor. She passed large amounts of urine. She stated that she drank at least 3 litres of water daily. The first examination of the radiographs revealed no abnormality apart from the fractures mentioned above. At the time of admission she showed considerable muscular hypotonia. Palpation of the bones of the extremities revealed swellings in several places. A mass the size of a hazelnut which might be a parathyroid gland was felt in the neck. The blood calcium was about 15 mg. per cent. X-ray investigation of the parts of the bones where abnormalities had been palpated revealed numerous rounded areas of rarefaction. There was no sign of serious renal damage. A straight X-ray of the kidneys showed no stone. There was albuminuria or a trace of albumin in the urine. The sediment showed only white cells. The non-protein nitrogen in the blood and the blood pressure were practically normal, 40 mg. per cent and 145/95 mm. Hg respectively. The fundi showed no signs of retinitis. The patient had pronounced polyuria and excreted as much as 5 litres per day. The quantity of calcium in the urine amounted to 1.0—1.6 gm. per day and that of phosphorus about 1.3 gm. per day. Deprivation of water did not affect the urinary output, neither did the administration of posterior pituitary extract. The temperature was slightly raised, the S. R. was 47 mm. per hour, and there was moderate anemia, Hb. 10

gm. per cent, R. B. C. 3.50 mil. per cu. mm., alkaline phosphatase 19 Bodansky units, citric acid 67 μ g. per cent.

She was operated in March 1945. The patient was thin and the region of the neck was explored without much difficulty. No definite parathyroid adenoma was found. A gland the size of two peas was found and removed. As there was a patient with tetany in the hospital at the time the gland was transplanted into an omental pouch of this patient who responded with a transient rise in blood calcium. It is therefore probable that what was removed was a parathyroid gland, possibly an adenoma. The operation did not produce any beneficial effect in our patient.

Three months later the patient was operated again. The surgeon palpated the upper part of the mediastinum but found no adenoma.

At that time it was not certain how the case should be classified from the point of view of pathological anatomy. The question as to whether there were adenomata or a diffuse hyperplasia of several glands was not settled.

It was considered, for the reasons given above, that, as no certain adenoma had been found during two operations, it was justifiable to try stilboestrol therapy. From Oct. 12th onwards the patient received 6 mg. of stilboestrol daily by mouth. The dose was later increased to 10 mg. This treatment was associated with a definite improvement in the patient's condition. She felt stronger. The muscular hypotonia disappeared. The blood calcium dropped but nevertheless remained above normal. The serum phosphorus rose from 2.0 to 4.2 mg. per cent. The amount of calcium in the urine fell to about half its previous value, 0.6—0.7 gm. Ca daily (see fig. 1).

The polyuria, however, remained. The excretion of phosphorus in the urine increased and in May 1946 it was 2.6—2.8 gm. per day. The non-protein nitrogen in the blood rose and at the beginning of Sept. 1946 it was about 64 mg. per cent. The albuminuria increased. There was no regression in the skeletal changes, nor was this to be expected for, since, as mentioned above, the excretion of calcium in the urine remained above

normal. The values for the phosphatase and citric acid were still raised, phosphatase 26—30 Bodansky units, citric acid about 90 µg. per cent.

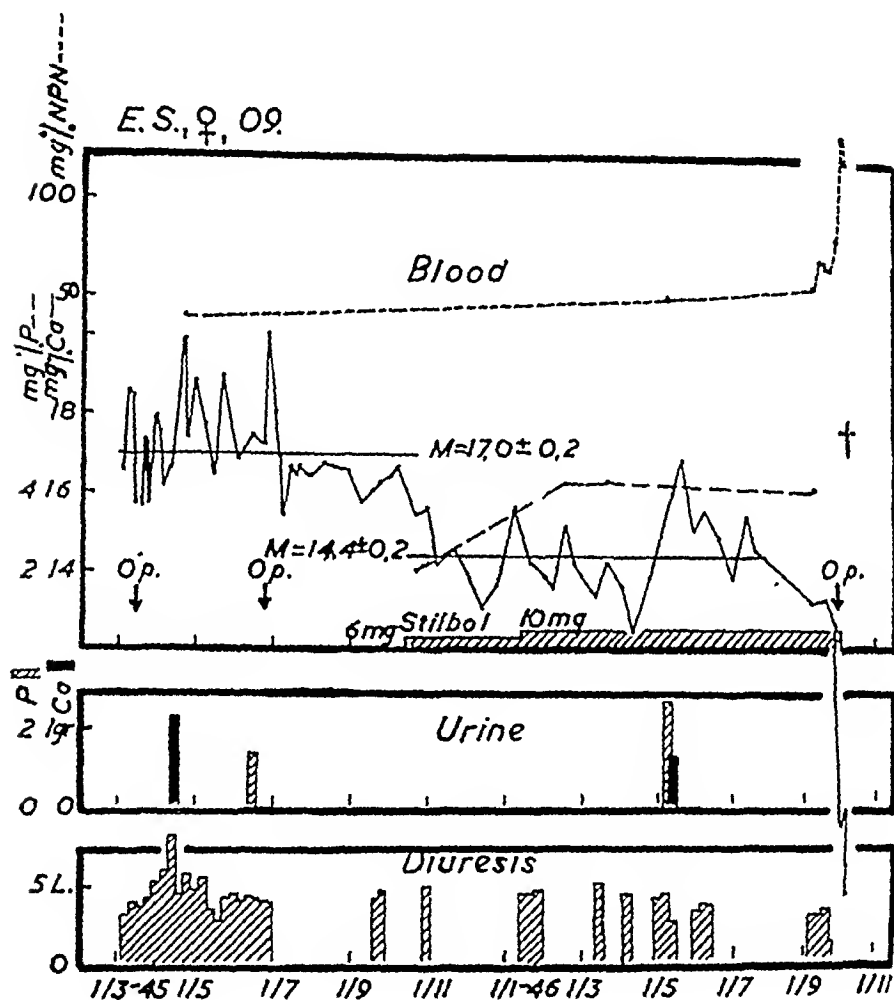


Fig. 1.

The patient was operated for a third time on Sept. 23rd. because of the rising value of the non-protein nitrogen in the blood. This time the surgeon performed a mediastinotomy, and found and removed a parathyroid adenoma the size of a haricot bean, lying behind the manubrium. The patient's reaction to the operation was only moderate. The day after the

Table 1.
Changes associated with the removal of a parathyroid adenoma.
E. S., ♀, 36 years of age.

Date	Urinary Output ml./day	N. P. N. mg. per cent	CO ₂ cap. vol. per cent	Serum Ca mg. per cent	Comments
9.9.46	3,000	67	41		
19.9	5,100	64		13.3	
20.9	3,900				
21.9	3,200				
22.9	3,700				
23.9 Operation	700			12.6	Vit.D ₂ 150,000 I. U. daily, 3 l. NaCl subcut., 400 ml. blood.
24.9	250	80		10.4	2 l. NaCl subcut.
25.9	550	92		9.0	Parathormone 100 U., 1½ l. isotonic sodium bicarbonate solution,
26.9	450	110	16	9.9	1½ l. NaCl.
27.9 Oedema	860	110	27	7.4	200 U., Ca-gluconate 2 gm. i. v., 4½ l. isotonic sodium bicarbonate solution.
28.9	760	110	35	7.9	Ca-gluconate 3 gm. i. v., 850 ml. blood.
29.9	710	134	40	6.5	4 gm. i. v., 300 ml. plasma.
30.9	520	120		5.8	5 gm. i. v., 300 ml. plasma.
1.10 Death					

operation, however, the urinary output had decreased to 250 ml. The non-protein nitrogen of the blood rose and, after three days, stood at 110 mg. per cent. Moreover acidosis supervened and the CO_2 capacity of the blood fell to 16 vols. per cent. The blood calcium fell and the patient had a couple of attacks of tetany. The urinary output and changes in the blood calcium are given in Tab. 1. The patient was treated with vitamin D_2 , parathormone, calcium salts intravenously and parenteral administration of fluids and sodium bicarbonate (see Tab. 1). She died a week after the operation showing symptoms of uremia and tetany.

The only points worthy of mention as regards the *post mortem* examination are that no remaining parathyroid glands were found; none of the glands examined microscopically were found to consist of parathyroid tissue; the kidneys showed areas of calcification and interstitial nephrosis; there was fatty degeneration of the liver.

As is seen from the description of the case given above, a definite improvement in the patient's condition occurred with stilboestrol therapy. She felt stronger. The muscular hypotonia diminished. The blood calcium fell and the blood phosphorus, which had previously been below normal, rose. The urinary excretion of calcium fell to about half its former value. The disappearance of the muscular hypotonia must be related to the fall in blood calcium. When the blood calcium level is raised the neuro-muscular irritability is diminished and there is secondary muscular hypotonia.

The undoubted improvement which occurred in the patient's condition obviously raised the question, whether the hyperparathyroidism had decreased.

The changes observed were as would be expected if parathyroid hyperfunction had regressed. The blood calcium fell, the excretion of calcium in the urine diminished, the serum phosphorus rose. Several points were, however, not explained. The polyuria persisted. The excretion of phosphorus in the urine increased. Later, the non-protein nitrogen in the blood began to rise and the albuminuria increased.

DISCUSSION

A review of the mode of action of parathormone is given here as it is relevant to discussion of the case which follows.

Administration of parathormone is followed by an increase in the calcium content of the blood and a decalcification of the skeleton. It has therefore been held that the action of parathormone consists partly in a direct effect on the skeleton leading to a breakdown and decalcification of the bones, and partly to an increase in the solvent power of the blood for calcium (*Thomson & Collip, 1932*). However, a more detailed analysis has shown that the matter is even more complicated (*Albright, 1929, 1941*). The first change which occurs after the administration of parathormone is an increased urinary excretion of phosphorus. The blood phosphorus decreases simultaneously. There is thus an increase in phosphorus excretion along with decreased blood phosphorus. In hypoparathyroidism the opposite occurs, i. e. a decrease in phosphorus excretion with an increase in blood phosphorus. It has been suggested that the primary effect of parathormone may be an effect on the kidneys leading to the increased excretion of phosphorus. The decrease in the blood phosphorus would lead to the mobilisation of phosphorus in the form of calcium phosphate derived from the skeleton. The hypercalcemia would thus be secondary to the hypophosphatemia. The hypercalcemia would then be followed by an increased excretion of calcium in the urine. As far as is known parathormone has no effect on the manner in which calcium is dealt with by the kidneys. The excretion of calcium in the urine depends on the blood calcium level, increasing when the blood calcium rises and decreasing when it falls. Administration of moderate doses of parathormone to bilaterally nephrectomized animals does not affect the blood calcium. This is also found after bilateral ligature of the ureters (*Tweedy et al., 1936, 1937*). If the lowering of the blood phosphorus under the influence of parathormone is prevented by the intravenous administration of phosphate, no rise in blood calcium occurs (*Neufeld & Collip, 1942*). The view has also been held that it

is possible to demonstrate, by means of clearance tests, that the reabsorption of phosphorus by the tubules is inhibited following the administration of parathormone (*Harrison & Harrison, 1941*). It seems that parathormone has a direct effect on the skeleton as well. The administration of large quantities of parathormone to nephrectomized animals raises the blood calcium and leads to decalcification of the skeleton (*Collip, 1934, Ellsworth & Fletcher, 1935, Selye, 1942, Stoerk, 1943*).

To return to the discussion of the present case: If the rise in the blood phosphorus shown by the patient had been due to a decrease of the hyperparathyroidism, it should have been followed by a diminished urinary excretion of phosphorus. The opposite was in fact observed. It appears to the author that the explanation of the changes which occurred must be sought elsewhere than in an alteration in the function of the parathyroid glands. The patient showed signs of progressive renal damage. Hyperphosphatemia may occur in chronic renal insufficiency.

If phosphate is added to blood *in vitro* it precipitates as calcium phosphate and the blood calcium falls (*Mc Lean & Hinrichs, 1938*). The fall in the blood calcium in the present case may have been due to an increase in the blood phosphorus secondary to renal insufficiency. The changes in calcium excretion and muscular tone are secondary to the lowering of the blood calcium. This does not necessarily mean that any diminution in the function of the parathyroid glands has occurred.

Operations on endocrine organs are occasionally followed by crises, and this has also been observed after the removal of parathyroid adenomata. Clinically they consist of hypocalcemia and tetany, as well as oliguria with a rising non-protein nitrogen in the blood.

The origin of the crises which are observed after operations on endocrine organs seems to be different with different glands. The crises which occur after removal of tumours of the suprarenal cortex are attributed to the tumour having produced excessive amounts of secretion, leading to atrophy of

the rest of the cortical tissue. Extirpation of such tumours leads to the development of acute supra-renal insufficiency, and the condition can be prevented by the administration of cortical hormone (*Walters, Wilder & Kepler, 1934*). The crises which occur after thyroidectomy are generally similar in their features to spontaneous thyrotoxic crises and are equally favourably affected by the administration of large quantities of iodine. The exact mechanism of these crises is not known (*Wijnblad, 1937, Bansi, 1939, Salter, 1940*).

In connection with the crises which occur after the removal of a parathyroid adenoma it is of interest to know whether or not there is an atrophy of the non-adenomatous glands. According to *Cope (1943)* the non-adenomatous glands are atrophic. This view is based on observations made at the time of operation. *Cope's* statement is, however, contradicted to some extent, by the observations of *Bergstrand (1921, 1931, 1938 and 1941)*. *Bergstrand* has made a histological examination of the parathyroid glands in a number of cases of generalised osteitis fibrosa and considers that there are hyperplastic changes even in those glands which are not the site of adenomatous change. The material was mainly obtained from *post mortem* examinations. As renal insufficiency is the commonest cause of death in generalised osteitis fibrosa, and as information is lacking as to the concentration of phosphorus in the blood, it is not impossible that the changes observed by *Bergstrand* were secondary to a hyperphosphatemia.

As a rule there is a fall in the blood calcium immediately after the removal of a parathyroid adenoma, and the patients often show signs of tetany. In exceptional cases the outcome may be fatal (*Beck, 1928*). The occurrence of oliguria as a post-operative complication has only been described in a few cases and there is generally no information in the literature about diuresis. However, *Snapper (1943)* states in a monograph that oliguria is a common, almost constant, phenomenon. Both the hypocalcemia and the oliguria disappear after one or a few weeks. In Table 2 the author has collected cases from the literature in which renal complications developed after the operation.

Barr & Bulger (1930), *Hellström* (1932), *Churchill & Cope* (1936) and *Snapper* (1943) amongst others have discussed the cause of postoperative tetany. The most obvious assumption is that it is due to the adenoma producing atrophy of the rest of the parathyroid glands (*Hellström*, 1932). However, as has been pointed out above, we do not know whether the other glands are in fact atrophic. *Barr & Bulger*, *Churchill & Cope* and *Snapper* consider that the postoperative tetany is due to the fact that after removal of the adenoma, the decalcified skeleton takes up calcium to such an extent that tetany sets in. *Barr & Bulger* (1930) described a case supporting this view. In this case the hypocalcemia was unaffected by daily injection of 100 units of parathormone, but was affected by intravenous injection of calcium chloride. The author has, however, found that the daily amount of parathormone required by a rabbit in order to maintain normal blood calcium and phosphorus levels after parathyroidectomy, is about 100 units when given by continuous intravenous injection. It is thus possible, that the amount of parathormone administered by *Barr* was too small.

In analysing the causes of postoperative tetany, account should be taken, not only of the blood calcium, but also of the phosphorus content of the blood, the excretion of calcium and phosphorus in the urine, and of the fact that parathormone affects the kidneys as well as the skeleton. In typical cases of generalised osteitis fibrosa there is a raised blood calcium, and a lowered blood phosphorus, as well as increased urinary excretion of both calcium and phosphorus. As a result of the operation both calcium and phosphorus practically cease to be excreted in the urine. The blood calcium may fall to below normal. The blood phosphorus remains mostly unchanged or rises.

The fact that phosphorus disappears from the urine in spite of a rise in the blood phosphorus must be considered in conjunction with the cessation of the inhibitory action of parathormone on the reabsorption of phosphorus by the tubules. Calcium phosphate is once more deposited in the skeleton, and

Table 2.

Cases of hyperparathyroidism with renal complications associated with the removal of parathyroid adenomata.

	Before operation							After operation						
	Urinary Output ml./day	N. P. N. mg. per cent	CO ₂ cap. vol. per cent	Serum Ca mg. per cent	Serum P mg. per cent	Urine		Urinary Output ml./day	N. P. N. mg. per cent	CO ₂ cap. vol. per cent	Serum Ca mg. per cent	Serum P mg. per cent	Urine	
						Ca mg. per 24 hrs.	P mg. per 24 hrs.						Ca mg. per 24 hrs.	P mg. per 24 hrs.
Gold 1928	700			13		400		250			10		25	
Snapper 1930	2,500	31	51	20	2.1	400	900	150	64		7.7			
Albert 1933	2,500		51	14	7			anuria		27	7			
Snapper 1943	700	54		16	2.0	50		100	180		7	3		
Hypovitaminosis D														
Snapper 1943	1,100	52 ¹⁾		16	2.8	250		115	93		9	1.8-2.7	20-40	50
Wildner & Howell 1936	7,000			16	2.5	1,000	1,000	250			6.5	2.5-4	50	
Moulouguet 1938	2,000	44 ²⁾		12	1.9	600- 1,200		anuria	180		7			
Couch & Robertson 1941	1,500	45	44	15	1.8	350			54 ³⁾	19	7	3.1		
Alexander et al. 1944 (Case VII)	3,000	4 ⁴⁾		15	2.6				78		7.8			
Törnblom 1949	3,500	65	41	13	4.2	650	2,700	250	134	16	5.8			

Post-operative values for urinary output CO₂ capacity, Serum Ca, ¹⁾ Urea-clearance 20 per cent of normal.
and Ca and P excretion represent lowest noted values, N. P. N. ²⁾ Urea-N.
³⁾ Post-operative hypertension.
⁴⁾ Reduced urea-clearance.

as a result of this the blood calcium falls. The blood phosphorus depends partly on the amount of phosphorus retained by the kidneys, and partly on the amount that is taken up by the skeleton. The decreased excretion of calcium in the urine is a result of the lowered blood calcium level. As phosphorus may be practically absent from the urine, even in normal subjects (*Smith*, 1937, 1943), the disappearance of phosphorus from the urine does not give any indication as to whether the parathormone content of the blood has fallen to normal or below. Hence it is no guide as to whether the non-adenomatous parathyroids are normal or atrophic.

The treatment of post-operative tetany consists in the maintenance of the blood calcium by the administration of calcium parenterally and *per os*, together with the administration of parathormone and AT 10 or vitamin D. The blood calcium and signs of tetany serve as guides of therapy.

The origin of the renal complications have been discussed by *Snapper* (1943). He attributes the oliguria to cessation of the diuretic effect of parathormone, and points out that the administration of parathormone to cardiac patients with oliguria often has a diuretic effect. As the oliguria following operation appears to be opposite in origin to the polyuria associated with generalised osteitis fibrosa, the latter will be discussed first.

Polyuria is common and may be abundant. A urinary output of 6—7 litres is not unheard of. The polyuria of generalised osteitis fibrosa has been compared to that observed in diabetes mellitus. The amount of glucose which can be reabsorbed by the tubules is limited. If, therefore, the concentration of sugar in the blood and glomerular filtrate is high, the reabsorption of water is prevented by the osmotic effect of the glucose. It has been thought that polyuria in generalised osteitis fibrosa was an osmotic effect produced by the large amount of calcium phosphate excreted in the urine. *Ask-Upmark* (1931), however, has suggested that the parathyroid hormone in itself exerts a diuretic effect on the kidneys. Table 3 is taken from a paper by *von Noorden & Isaac* (1927) and shows the rela-

Table 3.

Urinary output in ml./day	Per cent glucose
1500—2500	2—3
2500—4000	3—5
4000—6000	4—7
6000—10000	6—9

tionship between the diuresis and sugar content of the urine in diabetes mellitus. The patient here described excreted 1.0—1.6 gm. Ca and 1.3 gm. P when the urinary output was about 5 l. per day. A diuresis of 5 litres a day in diabetes mellitus corresponds to a sugar concentration of about 5 per cent, i. e. a 0.28 molar solution of glucose. If the calcium in the urine is present in the form of $\text{Ca}(\text{H}_2\text{PO}_4)_2$, CaHPO_4 and CaCl_2 and if these compounds are assumed to be completely dissociated, the urine must contain 4 gm. Ca per litre if the solution is to be osmotically equivalent to a 0.28 M glucose solution. There would thus be 20 gm. in 5 litres. However the salts mentioned cannot be entirely dissociated, so that there would, in fact, have to be even more calcium present in order to attain the osmotic effect discussed. Even taking into account the phosphorus in the urine, the polyuria in generalised osteitis fibrosa cannot be explained by an osmotic effect in which the calcium and phosphorus correspond to the sugar in diabetes mellitus. The polyuria of hyperparathyroidism must therefore be due to some diuretic effect of parathormone, which is not entirely brought about by the osmotic effect of the increased calcium phosphate excreted. The fact that the polyuria ceases after extirpation of the parathyroid adenoma means that there is no reason for assuming that the polyuria is due to any factor other than parathormone itself. Furthermore it is known that the administration of parathormone has a diuretic effect (*Taylor, 1926, Albright et al. 1929, Hueper, 1929*). The mechanism by which parathormone brings about its diuretic effect is not known. It is possible that

parathormone inhibits directly the reabsorption of water by the tubules.

It is reasonable that the polyuria should disappear when the parathyroid adenoma is removed. But why should oliguria occur? In the authors opinion the available findings give no definite explanation. It is possible that the normal amount of parathormone is necessary to maintain a normal urinary output. If there were atrophy of the non-adenomatous glands, oliguria would then occur, and might be accentuated by previous damage to the kidneys. *Thomson & Collip* (1932) state that in animals anuria may develop as a result of removal of the parathyroid glands. It is possible that the polyuria which occurred earlier gave rise to a compensatory functional change in the anterior and posterior lobe of the hypophysis which normally regulate the urinary output, and that this functional change remains after removal of the parathyroid adenoma, and thus gives rise to oliguria. If this were the case, there should be either an increase in the antidiuretic effect produced by the posterior lobe, or a decrease in the diuretic effect produced by the anterior lobe, or a combination of both. The oliguria following on the removal of a parathyroid adenoma persists for about a week. The regulating mechanism of the posterior lobe appears to act rapidly (*Brun, Knudsen & Raaschou*, 1944, 1945) and the effect of its antidiuretic substance to be of short duration. The diuretic effect of the anterior lobe is of longer duration (cf. *Forssman's* monograph 1945) and a diminution of the diuretic activity of the anterior lobe seems more probable than an increase in the activity of the posterior lobe.

A rise in the non-protein nitrogen of the blood occurs in association with the oliguria. This at least may be partly explained by the fact that reabsorption of urea by the tubules depends upon the amount of the urinary output (*Möller, McIntosh & van Slyke* 1928). If but little urine is excreted, the reabsorption increases. The rise in the non-protein nitrogen should make itself felt especially in cases where is a latent renal insufficiency. In such cases the rise in the non-protein

nitrogen has been counteracted by the polyuria before the operation.

In the case under discussion, acidosis occurred as well as oliguria and a rise in the non-protein nitrogen. One change which may well have contributed to the acidosis is as follows. Previously about $1\frac{1}{2}$ gm. of phosphorus per day had been excreted in the form of acid phosphates. Before the operation the pH of the urine had varied between about 6.3 and 6.4. After removal of the adenoma the excretion of phosphates ceased. It is further probable that the reabsorption of sulphate, like the reabsorption of urea, is dependent on the amount of urine being excreted, so that the oliguria was probably followed by retention of acid sulphates in the blood (*Hagman & Johnston, 1932, Macy, 1934*).

The above discussion suggests that the renal changes resulting from the removal of a parathyroid adenoma depend on the sudden disappearance of the diuretic effect of parathormone and may thus be prevented by the administration of parathormone.

Snapper (1943) states that it is usually possible to tide the patient over the post-operative oliguria by the intravenous administration of hypertonic glucose solution and the abundant subcutaneous administration of fluids. The effectiveness of this treatment must depend on the prevention of the water reabsorption in the renal tubules by making available sufficient glucose in the urine.

Of interest too are the questions when may these renal complications be expected, and when can they endanger life. If the above explanation of their origin is correct they should occur in cases of severe hyperparathyroidism. They should be especially dangerous in cases where signs of renal damage were evident before operation. The cases described in the literature showed either a high blood calcium with polyuria, or raised non-protein nitrogen in the blood or other signs of kidney damage before operation. Those cases in which the non-protein nitrogen rose above 100 mg. per cent had a raised non-protein nitrogen even before the operation.

Cases of generalised osteites fibrosa which were fatal on removal of parathyroid adenomata have been described by *Beck* (1928), *Wanke* (1930), *Ask-Upmark* (1931) and *Albert* (1933) (see above). Information as to the amount of urine excreted after the operation and the non-protein nitrogen in the blood is not given in these reports. *Beck* states, however, that in the case he described the CO_2 capacity of the blood was 52 vol. per cent before the operation and 35 vol. per cent just before death. *Beck's* and *Wanke's* cases showed signs of tetany, but the blood calcium never fell below normal. The only signs of tetany shown by *Ask-Upmark's* case were mental disturbances, motoric agility and a positive Chvostek's sign 9 days after the operation. The blood calcium fell below the normal value. No parathyroid tissue was found at autopsy in *Wanke's* or *Ask-Upmark's* cases. There is no information on this point in *Beck's* description. In none of the cases does it appear that the post-operative tetany was particularly marked. It seems impossible to determine from the available evidence, whether any renal complications, affecting the final outcome, occurred in any of these cases.

The blood calcium can be used as a guide for the treatment of postoperative tetany, which consists in the administration of calcium or calcium and parathormone. The renal complications do not seem to depend on the blood calcium and there is no reason to expect that they will be affected by the administration of calcium. Parathormone or intravenous hypertonic glucose solution should be given to counteract the oliguria, and the amount of urine excreted should be the guide for the treatment.

SUMMARY

A case of generalised osteitis fibrosa is described. The patient died showing signs of tetany and uremia, following the removal of a parathyroid adenoma. The patient had previously been treated with stilboestrol, apparently with some

success. The author is of the opinion that the grounds for believing that the improvement was apparent, and that it was caused by progressive renal insufficiency. An attempt is made to analyse the original renal complications with reference to the case as well as to those found in the literature in which renal complications occurred following removal of a parathyroid gland. In agreement with the suggestion of Snapper the author considers that they are due to the sudden cessation of the effect of the parathyroid adenoma, and that they are prevented by the administration of parathormone or 10% tonic glucose solutions given intravenously. The blood urea should act as a guide for the treatment of post-operative renal complications and the output of urine as an indication for the treatment of renal complications. It is suggested that renal complications are to be expected in cases where the hyperthyroidism is severe or where signs of renal damage are present before the operation.

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ANNOUNCEMENTS

from the Endocrinological Societies

FINNISH SOCIETY FOR ENDOCRINOLOGY

Meeting, March 31, 1948.

H. Hortling: Adrenals and hematopoiesis.

Insar Uoti: On the difficulties involved in the diagnosis of myxedema in children.

Meeting, Sept. 20, 1948.

P. Tuovinen: The hormonal back-ground of carcinoma of the prostate.

Olavi Kinnunen: Effect of the sulfonamides and methyl thiouracil on the blood iodine and blood cholesterol values in the rat.

Meeting, Nov. 30, 1948.

Johs. Wahlberg: The thiouracil derivatives and their use.

Eila Kalliokoski: On the relation between the adenohypophysis and the parathyroid glands, on account of a case.

Meeting, Febr. 7, 1949.

Reino Pohjola: The hormonal influence of orchietomy and orchididymectomy in rats, using the prostate gland as end-point of assay.

Carl-August Hernberg: Morbus Albright.

A. Pekkarinen: On the presence of adrenalin and its variations in the animal organism.

From the Laboratory for Electron Microscopy of the Nobel
Institute for Physics (Professor Manne Siegbahn) and the
Endocrine Department of Serafimerlasarettet (Rolf Luft, M. D.),
Stockholm, Sweden.

SUBMICROSCOPIC CYTOPLASMIC GRANULES IN THE ANTERIOR LOBE CELLS OF THE RAT HYPOPHYSIS AS REVEALED BY ELECTRON MICROSCOPY

BY

HUMBERTO FERNÁNDEZ-MORÁN and ROLF LUFT

This paper is a report on the preliminary results of a study of the glandular lobe cells of the rat hypophysis obtained by combining conventional cytological techniques with various electron microscopic methods.

The anterior hypophysis of the rat contains chromophobe, acidophile and basophile cells in approximately the same proportion as the human hypophysis. The normal cytology has been most thoroughly studied by *Nukariya* (1926), *Severinghaus* (1933), *Romeis* (1940), *Wolfe and collaborators* (1933–38), and by others. The relationship between the different cell types is the subject of numerous hypotheses (*St. Remy* 1892, *Erdheim* 1903, *Kraus* 1913, *Collin* 1928, *Severinghaus* 1933, *Franck* 1937, and others). All these theories deal with the specific granules, mitochondria, Golgi bodies and other structures seen with the light microscope. The electron microscope with its one hundred fold higher resolving power should contribute significantly to our knowledge of the sub-

microscopic organization of these cells by revealing structural details lying beyond the boundary of light microscopy. Its first application to this problem discloses the presence of a new submicroscopic structure which predominates in the cytoplasm of all cell types of the anterior lobe, and is represented by spherical granules which are approximately ten times smaller than the specific granules.

MATERIAL AND METHODS

At present there are few suitable techniques for the examination of cells with the electron microscope. Examination of an object with the electron microscope requires that it be ultrathin (0.1 micron or less), contain sufficient contrast and be subjected to a high vacuum. Since sections of the pituitary tissue could not be cut thin enough, it was necessary to use smears of glandular lobe cells on metal supporting membranes or on specially prepared glass slides, in addition to other procedures like isolation of the cells or maximal extension of fresh cellular sheets on a liquid surface (*Fernández-Morán*, 1948).

The anterior lobes of the hypophyses used in this study were taken from 50 mature albino rats (nearly all males) of an inbred strain. The rats were rapidly decapitated, and the pituitary glands immediately removed. The anterior lobe was then carefully separated from the rest of the gland and either smeared directly on very clean, »flamed« glass slides or on the metal or collodion supporting films of the electron microscope object holders. Several anterior lobes were frozen on the stage of a freezing microtome and »serial touches« prepared by pressing a clean glass slide gently on the tissue. Extremely thin patches of the gland adhere to the slide and can be aligned serially. Other anterior lobes were fixed *in toto* in neutral formalin (10 %) or in *Mellgren's* (1944) special fixation fluid, and used for mechanical disintegration procedures in order to obtain single cells or cell fragments. The smears and »touches« were either air dried without fixing, frozen dried or fixed with osmium vapor (from a 2 % neutral solution), formalin vapor or liquid formalin (neutral, 4 % and 10 %).

The serial smears or touches on glass slides were divided into 3 groups. One group was subjected to electron microscopical methods, and the other two were used for control staining with conventional microscopical techniques or for heavy shadowing with metals (ref. *Williams & Wyckoff*, 1946). By covering thin cell smears with a metal film of suitable thickness deposited obliquely in a

vacuum, those surface structures are brought out in clear detail which are close to the limit of resolution of the light microscope. The characteristic »bas relief« pictures obtained by this method are very similar to the electron micrographs of the shadowed specimen.

Preparation Techniques for Electron Microscopy.

All observations were made with the electron microscope designed by Prof. Manne Siegbahn at the Nobel Institute for Physics (1939). This laboratory model operates at 35–55 kV, attaining a resolving power of 20–50 Å. U. The procedures used in this study for examining the cellular material can be divided into 3 groups: (a) Examination of cell smears or isolated cells deposited on glass slides, by replication and repeated stripping, referred to here as the »Pre-shadow Replica-Adhesion Method« and its variations; (b) direct examination of the cell smears or extended cell sheets deposited on the specimen films of the object holders of the electron microscope; (c) plain incineration of the extended cells and replica-incineration methods.

(a) *The replica-adhesion technique.* This is a modification of the pre-shadowed replica method of Williams & Wyckoff (1944–46). The cells extended on a thoroughly cleaned, polished and »flamed« glass slide are covered with a thin 20–50 Å. U. uranium or gold film deposited obliquely (angle 5°–10°) in a vacuum chamber. This heavy metal film is reinforced by an equally thin aluminium or beryllium film deposited vertically or obliquely in the opposite direction. The combined metal film is now backed with a thin collodion membrane and stripped off under water. If the glass slide has been properly cleaned, the cellular material will stick firmly to the glass surface. When the collodion membrane is stripped off, only the metal replica and a very thin section of the cell surface are removed. The procedure can be repeated many times on the same cells, and each stripping will remove another ultrathin layer (0.1–0.03 micron thick) in addition to the metal replica of the exposed, new surface. The collodion metal film is mounted on the grids of the object holders, and examined directly with the electron microscope or placed previously in a bath of amyl acetate which dissolves the collodion, making the preparation thinner and more suitable for examination.

(b) *Direct examination of isolated cells.* These methods are mainly of interest as control procedures for the other two groups, since only the cytoplasm can be clearly seen, while the nucleus and the other dense components of the cell are too thick for electron microscopy.

(c) *Microincineration methods* (Fernández-Morán, 1948). A very thin extension of cells deposited on an aluminium or beryllium specimen film can be thoroughly incinerated with a microflame or in an electric oven at 500° C. Fractional incineration gradually clears up the dense areas of the preparation which pass through various characteristic transition stages, resulting finally in the formation of a residual »ash skeleton« of the cell. These »electron spodograms« are reproducible and can be used to identify a given cell type.

Replica-incineration (Fernández-Morán, 1948. Cells lying on a metal supporting film (25 Å. U. aluminium) are covered vertically or obliquely with another aluminium or beryllium film (20—80 Å. U.). Incineration is then carried out with the microflame or in an electric oven. The upper metal film which forms a cast of the cell surface is not appreciably altered during this incineration process. The cellular material, enclosed between the two metal films, is rapidly burned away, leaving residual ash deposits which are kept in place by the submicroscopic chambers formed by the metal envelope. A combination of the spodogram and the metal replica of the surface is thus obtained, showing both the surface structures and the residual ash contained within the cell. This method served as a control procedure in the study of well delimited, submicroscopic elements which cannot be touched by mechanical means, but may thereby be incinerated »in situ« giving some indication of their composition.

OBSERVATIONS

1. *Optical Microscopy.*

Smears or serial touches of the anterior lobe show rows of nuclei embedded in a sheet of granular cytoplasm. No cell boundaries are discernible, but closer examination reveals that each nucleus is surrounded by a characteristic »cell territory«. Thus, the large basophile cells are prominent because of their large nuclei, vesicular Golgi body and the numerous floccular mitochondria and vacuoles in the cytoplasm, while the eosinophiles and chromophobes can be made out in suitably stained preparations. If the smears are covered obliquely or »shadow cast« with a thick film of aluminum, the surface contrast is greatly enhanced and the smallest particles emerge clearly from the silvery background by the shadows they pro-

ject. It is then seen that the cytoplasmic ground substance which generally appears homogeneous is sprinkled with minute particles (not larger than 0.3 micron), evenly distributed among the larger specific granules.

2. *Electron Microscopy.*

1. *Direct examination of extended cells.* Smears of air dried or fixed glandular lobe cells placed on collodion or metal supporting films are thin enough, in certain areas, for electron microscopy. The cytoplasm surrounding the dark nuclei consists of dense spherical or irregular particles of 0.3 to 0.05 microns embedded in a still finer, granular ground substance. The large specific granules stand out clearly among bulky bodies adjacent to the nuclei. Prolonged microincineration of these specimens does not change the picture significantly. Every spherical granule leaves a residual ash particle, and these keep their original position forming a regular network pattern.

2. *Examination of the surface replica-adhesion preparations.* This method gives a plastic image of the cell surfaces from the different layers stripped off. If the minute granules seen with the light microscope in the cytoplasm after shadow casting are followed up with the electron microscope, it is found that they represent the largest bodies among a profuse array of spherical elements. *The cytoplasm appears as a dense heap of massed spheres arranged in clusters or acini.* These spherical bodies are sharply outlined and appear uniformly round and firm. Most of them are beyond the resolving power of the light microscope, with a diameter ranging from 30 to 300 millimicrons (figs. 1, 2, 3, 4). The cytoplasmic spheres are embedded in a particulate ground substance which consists of smaller spherical granules reaching sizes down to the limit of resolution. The perfectly round shape of the cytoplasmic spheres often suggests the presence of a membrane, while others are covered with small round granules or pits. The specific granules, although five to ten times larger (fig. 3),

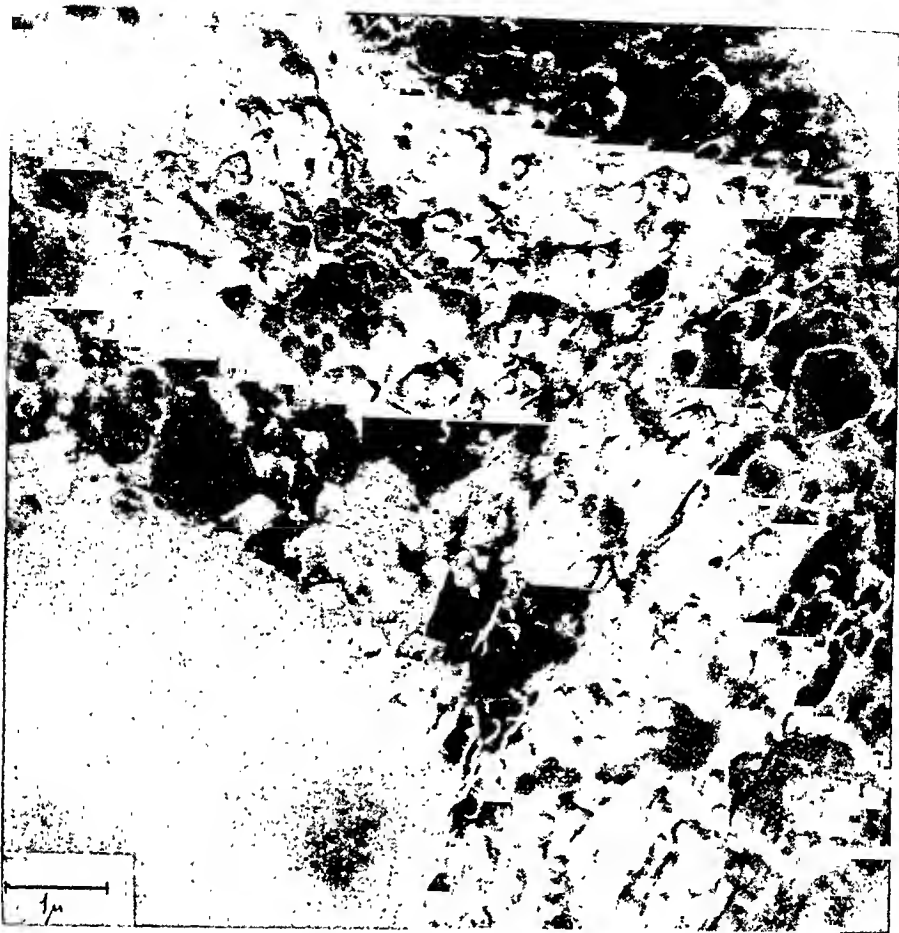


Fig. 4.

Electron micrograph of a segment of a large basophile cell from a smear of the anterior lobe. The dense nucleus is surrounded by massed collections of spherical bodies of 50 to 300 millimicrons in diameter. Preshadowed replica-adhesion method (gold/aluminum). Magnification 11500 \times .

show a certain similarity to these submicroscopic granules. These spherical elements build up almost exclusively the cytoplasm of the glandular lobe cells of the rat hypophysis, giving the different cell types a common submicroscopic appearance. The most common types of spheres found in the cytoplasm of the glandular cells have an average diameter of

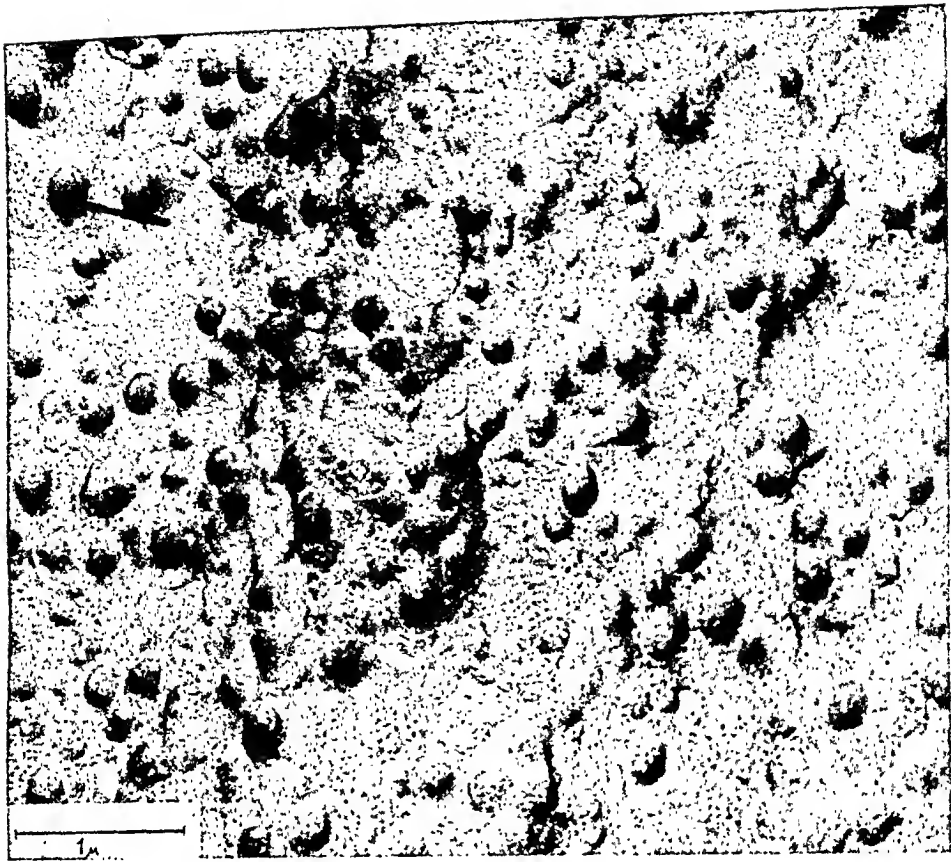


Fig. 2.

Section of the cytoplasm of a large basophile cell from a thin smear of the anterior lobe. The cytoplasmic spheres are spread and their full round shape is more distinct. Metal surface replica obtained by the replica-adhesion-method. Electron micrograph. Magnification 19000 \times .

100—250 millimicrons. They will be referred to here as »cytospheres« or »endospheres« to distinguish them from the specific granules of light microscopy. The smallest cytospheres are generally found in the immediate vicinity of the nuclei or on top of the nuclear membrane, while the larger ones are more abundant in the periphery of the cell. They are dense and impenetrable to the electron beam in ordinary preparations, but preshadowed replicas (using gold or uranium) reveal extremely small, uniform microgranules (of 2—20 millimicrons) arranged in a regular pattern on their surfaces. The

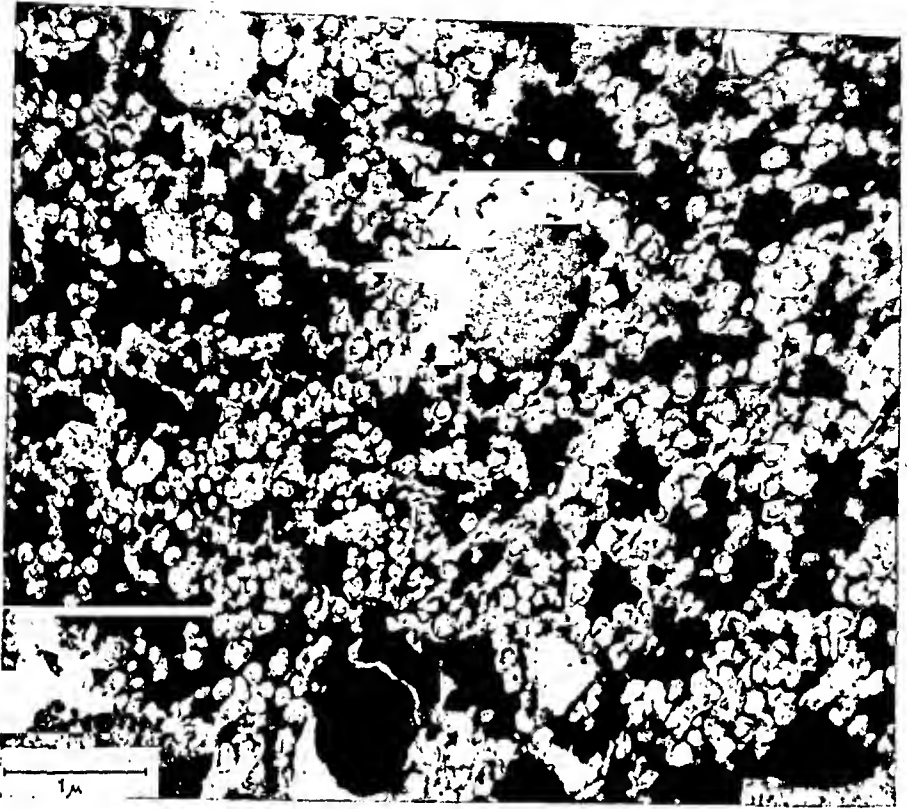


Fig. 3.

Cytoplasm segment in an eosinophile cell of the anterior lobe. Two opaque oval bodies, corresponding to the specific granules, are embedded among the closely packed cytoplasmic spheres, which are approximately ten times smaller. Notice the finely granular membrane of the specific granules. Metal replica-adhesion method. Formalin fixed smear (gold/aluminium). Magnification 16000 \times .

possibility of metal granulation artefacts which are often encountered in these preparations detracts from the value of this finding. However, when the cytospheres are disintegrated by mechanical procedures, minute spherical particles of the same dimensions are obtained. After prolonged incineration every cytosphere leaves a residual round or cubic ash particle, surrounded by hardly visible, smaller ash particles.

The specific granules (fig. 3) range in size between 0.5 and 1.5 microns and are round or oval masses bounded by a mem-



Fig. 4.

Portion of cytoplasm of a large basophile cell. The empty surface cast of an irregular oval body, dotted with coarse granules, is seen among large accumulations of cytoplasmic spheres. These bodies may correspond to the large floccular mitochondria and appear clearly in preparations dissolved with acetone or amyl acetate. Replica-adhesion method. Air dried smear. Magnification 16000 \times .

brane impregnated with minute spherical particles. Their core consists of dense round bodies which change into sharp crystalline ash particles after prolonged incineration.

In addition to these spherical particles large irregular bodies or vacuoles are seen in the cytoplasm, particularly in the large basophile cells. They are irregular or indented polygonal bodies of 1—4 microns which stand out clearly in preparations dissolved with amyl acetate or acetone (fig. 4). These bodies appear to correspond to the large floccular mitochondria described by *Severinghaus* (1933), but they have not been definitely identified. The same holds true for the correlation of the large spherical masses or vacuoles, lying next to the nucleus, with the Golgi body of classical cytology. *Severinghaus* (1933) describes the Golgi body as a »hollow sphere with one or more invaginations« enclosed in a membrane. The larger juxtannuclear bodies seen in electron micrographs fit this description as regards location, size and the presence of a characteristic surface structure which resembles a membrane.

The nucleus. Only the surface of the nucleus could be adequately studied since the firm nucleus resists all extension and stripping methods and is too dense for microscopy. The nucleus is sharply outlined and covered with a creased and shrunken membrane. This shrinkage does not obliterate the dense conglomerates of spherical bodies of various sizes, predominantly small ones (20—50 millimicrons) which seem to lie below the membrane and apparently belong to the superficial layers of the nucleus. Very often these spheres, which are identical in size and shape to the cytospheres, give the impression of emerging from beneath the nuclear membrane by a process of extrusion.

A survey of the electron microscope observations at this preliminary stage indicates that there are no basic differences in the submicroscopic structure of the three principal cell types, since all of them contain cytospheres which constitute the bulk of their cytoplasm. The differences encountered seem to be rather of a quantitative nature, involving the size and distribution of the cytospheres and the configuration of the juxtannuclear vacuole and of the lipoid soluble bodies. This

applies of course only to the morphological picture as shown at this dimensional level by the electron microscope. The methods employed here do not allow of a chemical identification of the structures, but further analysis with adequate procedures may make a closer correlation possible with the cell types recognized by classical methods.

All the cytoplasmic elements described in the preshadowed replica-adhesion preparations are seen in the control preparations produced with other methods. Electron microscopical examination of mechanically disintegrated anterior lobes fixed in formalin shows a predominance of spherical and oval elements of the same size and shape as the cytospheres, covered with tiny spherical elements.

DISCUSSION

An interpretation of these preliminary findings obtained with new methods, which are still in an early stage of development, and have not been adequately tested, must necessarily be tentative in nature.

The techniques of preparations used, obliterate cell boundaries and introduce arbitrary concentrations of cytoplasm, and they are, moreover, subject to numerous possibilities of artefact formation such as fixation artefacts in the cells, distortions in the replica films, the effects of a high vacuum and electron bombardment, etc. Certain general findings, however, are confirmed by different methods of examination, and can be considered here.

The observations with the electron microscope disclose a great structural similarity between the basic constituent elements of the anterior lobe cells, which show a remarkably uniform submicroscopic organization. The light optically »clear« cytoplasm is seen to consist of well defined spherical elements, the cytoplasmic spheres or cytospheres, which vary in size between 30 and 300 millimicrons and are thus beyond

the resolving power of the light microscope. The other cytoplasmic constituents, like specific granules, juxtannuclear (Golgi) body, etc. have also a similar submicroscopic structure in the different cell types recognized with conventional cytological methods.

The preponderance of one common element and the apparent similarity in structure may point to a close relationship between the different cell forms as regards functional states and cell origin. The constituent material of the cytoplasm appears to be divided into sharply outlined and progressively smaller units (specific granules, cytoplasmic spheres, submicroscopic ground substance) and it remains to be investigated in which way they are related to each other.

SUMMARY

A study of the glandular lobe cells of the rat hypophysis with the electron microscope, using different techniques of preparations (mainly the replica-adhesion method carried out on cell smears), shows that the cytoplasm of all glandular lobe cell types examined consists predominantly of distinctly outlined spherical bodies of 30—300 millimicrons in diameter. These »cytospheres« contain smaller spherical granules and are embedded in a particulate submicroscopic ground substance. The specific granules appear as large spherical bodies bounded by a membrane with fine adhering granules. Small spherical bodies resembling the cytospheres are found in large numbers attached to the corrugated nuclear membrane. No fundamental differences in the submicroscopic structure of the three basic cell types could be observed.

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ATTEMPTS TO INDUCE EXPERIMENTALLY MATURATION OF THE GONADS OF THE EUROPEAN EEL, *ANGUILLA ANGUILLA* L.*)

BY

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INTRODUCTION

In 1904 the Danish marine biologist Dr. *Johannes Schmidt* found the first eel-larva in the Atlantic Ocean (*Petersen*, 1905). This discovery became the starting-point for extensive marine biological investigations and made it possible to prove that the European Eel breeds in the early spring in the Sargasso Sea, presumably at a depth of 200—500 m.

Ripe eels' eggs have never been observed with certainty, but the youngest eel-larvae ever seen, the so-called Leptocephali, are 6 mm. long, and most probably only a few days old. In the course of 2½ years the eel-larvae drift with the Gulf-Stream and the North Atlantic Current towards the coasts of Western Europe, gradually attaining a size of 70—80 mm. In the deep water off the coasts, the larvae metamorphose into elvers and then cross the continental shelves to get into the brackish water areas or to ascend the streams and rivers.

The small glass eels or elvers which we see every spring near our shores in great numbers are thus three years old,

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and during these three years they have travelled about 5000 km. They tend to swim against water currents and towards less salty water (Sylvest, 1931), and thus gradually get into brackish water or fresh water, where little by little their migration urge disappears though often not until after a couple of years.

When the elvers have reached the coasts and are in fresh water, a pigment begins to deposit in their skins, and in the course of their fourth summer they assume the well-known appearance of small eels, viz. dark olive-greenish above and yellowish-white on the abdomen: they have become yellow eels, and now live as benthonic animals and have a ravenous appetite.

When they have spent at least 6—7 years as yellow eels they again change their appearance and become silver eels; that is, the back and the pectoral fins turn almost black, the sides and ventral surface assume a silverish metallic lustre, and the eyes grow larger. Changes also occur in the internal organs: the gonads increase in size, the intestinal function ceases and in the digestive canal atrophic changes set in.

The silver eels have a migratory urge. They migrate downstream and towards salt water. During the three autumnal periods of moonless nights they leave our shores at a speed established by marking experiments to be up to 52 km. per 24 hours. The last we know of the silver eels originates from a few fishings in the deeper part of the Channel and from some half-digested remnants from the stomach of a Sperm Whale from the Azores. But the goal of the silver eels we know; they are bound for the Sargasso Sea »pour l'amour et la mort« as once stated by a *spirituel* Frenchman. When they have bred they die — if one may judge from related forms; at all events they never appear again.

We may consider it established that normally the two sexes are differentiated when the eels attain a length of 18—26 cm. Until then it is impossible to tell whether an eel is a female or a male.

The metamorphosis from yellow eels into silver eels takes

place in the male eels at a length of 29—40 cm., and the length of a male silver eel never exceeds 50 cm. The females do not become silver eels until they are some 42 cm. long and as a rule not until they are more than 50 cm. long. This thus means that if a silver eel is longer than 50 cm., it is a female; and if it is less than 42 cm, it is a male.

The testes of the silver eel consist of a pair of grayish thread-shaped, somewhat lobed organs, the diameter of which hardly exceeds 1.5 mm. They extend throughout the length of the abdominal cavity. Microscopically small groups of rather big cells rich in protoplasm with nuclei rich in chromatin are found in the connective tissue stroma. These cells are resting spermatogonia.

The ovaries of the silver eel consist of a pair of 5—20 mm. broad, curly or plaited bands extending along the whole length of the dorsal side of the abdominal cavity. The ovary contains exclusively egg cells kept in place by a little fibrous tissue. The cells are all of nearly the same size, and their appearance suggests that in any single ovary all egg cells are at the same stage of development. Marine-biological investigations of a large number of ovaries from silver eels, show that the average egg is between 0.16 and 0.18 mm. in diameter. The protoplasm contains numerous fat droplets. The eggs of the yellow eel are on an average 0.09 mm. in diameter (e. g. *Walter*, 1910), and in these no fat droplets are found.

This is a brief account of all that is known regarding the sexual biology of the European Eel. For the sake of completeness we may add that a few eels have been observed, in which the degree of maturity was remarkably more advanced than is normally the case. Thus *Schmidt* (1906) described a male silver eel whose testes were mature, and similar observations have been made by *Grassi & Calandruccio* (1897) and *Rodolico* (1933). *Calderwood* (1893) described a female eel so nearly sexually mature that the eggs apparently were quite ready to drop from the outer surfaces of the ovaries, which showed no signs of any blood supply, and when touched crumbled away very easily. The nuclear membrane in each ripening

ovum was, however, still visible in sections. It is regrettable that he did not state the diameter of the eggs. However, *d'Ancona* (1939) mentions a female silver eel with egg cells having a diameter of up to 0.31 mm. But these examples are so extremely rare that they cannot by any means contribute to dispelling the mystery which surrounds the period of reproduction of the European Eel.

Attempts at producing sexual maturity in the European Eel were first published by *Rodolico* (1933), who placed male silver eels at the hydrostatic pressure to which he supposed the eel to be subjected in the Sargasso Sea, viz. a pressure of 10 atmospheres. This corresponds to a depth of 100 m., which he supposed to be the depth at which the Eel breeds. The result of these experiments was that the testes underwent degenerative changes. Experiments of a similar kind and giving the same result were published by *Beccari & Baldasseroni* (1936).

Boucher, Boucher & Fontaine (1934), *Fontaine* (1936), and *Tuzet & Fontaine* (1937), were the first to publish results of experiments in which gonadotrophic hormones were injected into the Eel. In several cases they succeeded in producing complete spermatogenesis in male silver eels by injection of urine from pregnant women. Similar experiments with a female silver eel gave no really positive results.

The results of *Fontaine & co-workers* have been confirmed by *Schreiber* (1935a, 1935b, 1936, 1937a, 1937b) using chorionic gonadotrophin as well as by *van Oórdt & Bretschneider* (1941) who used pituitary extracts from mammals and sexually mature carps as well as other gonadotrophic substances.

Ira Hansen (1939) reported that treatment of female *Anguilla rostrata* (Le Sueur) (the American Eel) with injections of the chorionic gonadotrophin Antuitrin S (Parke, Davis & Co.) and of suspension of frog's pituitary gland had no effect on the ovaries.

OUR OWN EXPERIMENTS

In 1941 the authors communicated in the Biological Society of Copenhagen (see *Bruun & Møller-Christensen*, 1941) some preliminary results obtained with hormone injections into the European Eel; and these results are incorporated in the present paper. *Hemmingsen* was in the Far East during the war.

From 1930 to 1940 we worked with female eels only and had no positive results to show. We shall only mention that besides giving intramuscular injections of chorionic gonadotrophin we also performed a large series of transplantations of pituitary glands from ox, swine, rat, garfish, plaice and cod, into female eels.

From 1940 we had ideal working conditions at the then recently built »Danmarks Akvarium«, Charlottenlund, Copenhagen, and we now started a series of extensive experiments.

From January 1940 to June 1947 the experiments comprised 7 male and 99 female silver eels.

As far as possible we have tried to keep the hydrogen ion concentration of the sea water used at 8.0—8.2, a salinity of 3.5 per cent, and a temperature of 18° C., i. e. near the values obtaining in the depths of the Sargasso Sea at which the eel is supposed to spawn. The illumination of the experimental room was faint daylight.

We have used for hormone treatments the chorionic gonadotrophin preparation »Physex Leo«, which is manufactured from human pregnancy urine, and the synthetic non-steroid oestrogenic substance, 4:4'-dihydroxyphenyl- γ - δ -n-hexane as manufactured under the name »Hexoestrol A. B.«. The chorionic gonadotrophin was injected into the female eels intraperitoneally in aqueous solution almost always in doses of 500 I. U., generally once a week; and Hexoestrol was given intramuscularly in a solution in arachid oil almost always in milligram doses, also usually once a week.

The male silver eels received chorionic gonadotrophin only, intraperitoneally, but in considerably smaller doses than the females, viz. from 400 to 800 units, spread over 8 to 20 weeks. In 3 of the males full spermatogenesis resulted, and a milky

fluid rich in spermatozoa could be squeezed out of the cloacal aperture. In the other males a distinct effect was also seen both microscopically and with the naked eye (see Fig. 1).

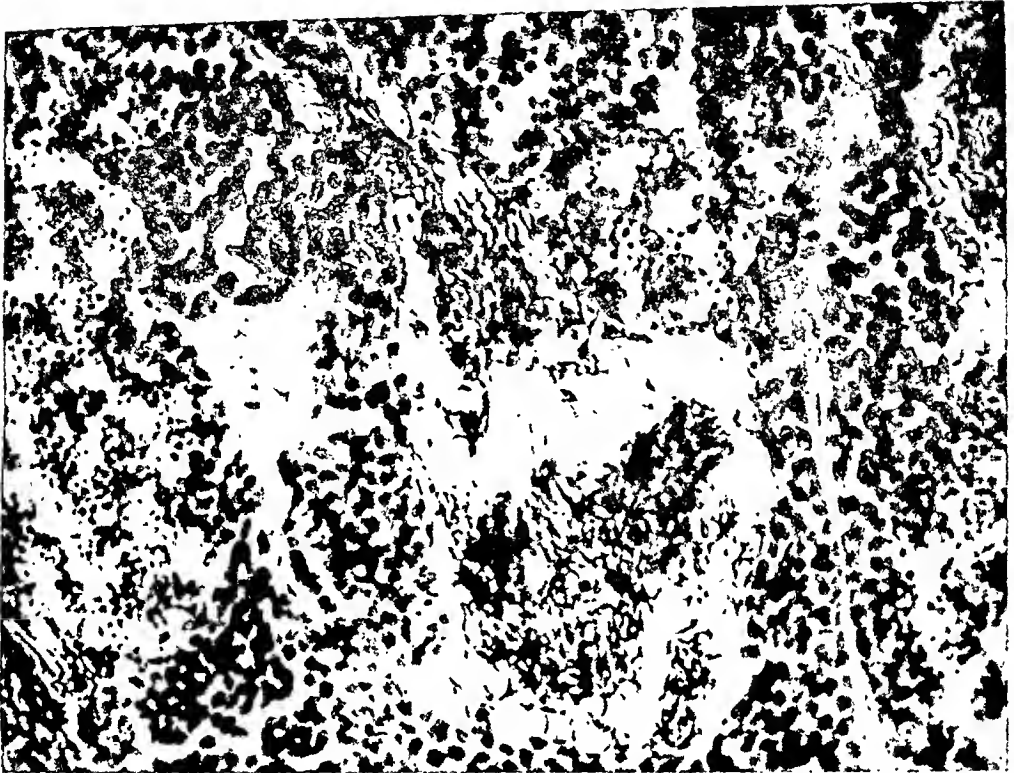


Fig. 1.

Ö. Winge.

Section of testis of male silver eel injected with 750 I. U.
of Physex.

The female silver eels were divided into two groups, one receiving chorionic gonadotrophin (Physex) only, the other one Physex + Hexoestrol. The effect of the hormone was studied by measuring the egg diameter (average of maximum and minimum diameter in each of 10 eggs chosen at random). The ovaries were fixed in 4 per cent formalin, and afterwards the measurement of the egg diameter was made by means of *Edinger's* projector.

On account of black-outs, curfews, and other inconvenien-

ces caused by the war, some measurements could not be made until a long time after fixation. A certain amount of shrinkage (perhaps 15—30 per cent of diameter) may therefore have taken place, so that our figures are minimum figures. Some material has been lost owing to a fire which occurred after 1941.

One of the most serious difficulties met with in our experiments was the high mortality of the eels. It was considerably reduced by adding to the sea water 2 g. euflavine (trypaflavine) per m³. Still the duration of many experiments was limited by the death of the eels.

In the animals which received Physex only, a total amount of up to 11500 I. U. has been given and the experiments lasted from 3 to 21 weeks. In the eels which received Physex + Hexoestrol the total dose varied from 1500 I. U. of Physex + 3 mg. Hexoestrol to 12500 I. U. of Physex + 25 mg. of Hexoestrol, and the experiments lasted from 3 to 42 weeks. The duration of the experiments includes the period of time elapsing from the last injection till the eel died or was killed (in some cases up to 3—6 weeks).

Table 1 shows the egg diameter of our own control (untreated) silver eels.

Table 1.
Untreated control female silver eels.

Nr.	Egg diameter
I	0.18 mm.
II	0.12 "
III	0.13 "

According to published records the diameter of the eggs of silver eels does not exceed 0.3 mm. (*d'Ancona*: 0.31 mm.), and usually is between 0.1 and 0.2 mm. (*Robin*, 1881, *Möbius*, 1887, *Jourdain*, 1889, *Williamson*, 1895, and *Walter*, 1910). Measurements given by *Möbius* (1887) and *Williamson* (1895) together with our own, and *d'Ancona's* exceptional record, are shown as control measurements to the left in Figs. 2 and 5.

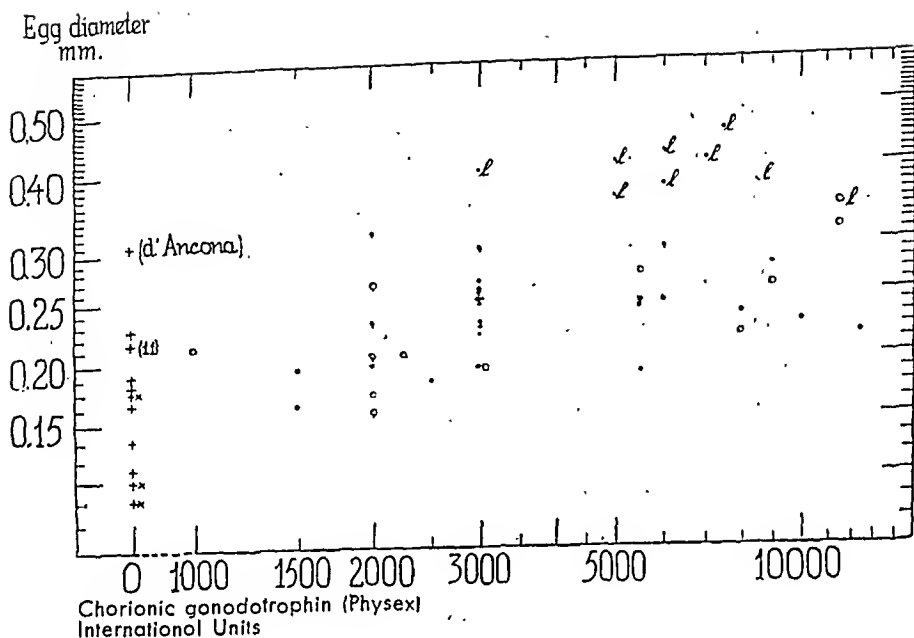


Fig. 2.

Correlation between egg diameter of silver eels and total dose of chorionic gonadotrophin (Physex Leo) injected intraperitoneally.

+ Controls (from Möbius, 1887; Williamson, 1895; and d'Ancona, 1939).

× Own controls.

● Physex + Hexoestrol (with very few exceptions 1 mg intramuscularly per 500 I. U. of Physex). Injections started autumn-January.

● Same, but injections started February-April.

○ Physex only. Injections started autumn-January.

○ Physex only. Injections started February-April.

l Eggs loose.

In Fig. 2 the egg diameters are plotted against the total doses of Physex injected. Only eels in which less than six weeks have elapsed between the last injection and death, are included. The eggs of eels killed later (6—22 weeks after the last injection) all measured 0.15—0.30 mm. in diameter, and there is reason to suspect that they had decreased in size since the last injection. Those injected with Physex only and those injected with Physex + Hexoestrol, are shown differently.

With very few exceptions the dose of Hexoestrol was varied with the dose of Physex (1 mg. Hexoestrol per 500 I. U. of Physex).

Logarithmic ordinate and abscissa axes have been chosen, because 1) the egg diameters obtained with any given dose appeared to have a skew distribution with a dispersion rising with dose, whereas their logarithms were more normally distributed with more uniform dispersion at all dose levels (for this kind of frequency distribution, see *Hemmingsen*, 1934); 2) the relation between log. effect and log. dose may be at least roughly linear; 3) the correlation is illustrated much more clearly in the graph than it would be if the actual figures were plotted. Injections were usually started in November-January. Some, which started in February-April, are marked specially in the figure. There is some indication in these latter experiments that a certain increase in egg diameter was produced by smaller doses of the hormone. It will be seen that, both with and without Hexoestrol, a positive correlation is evident between egg diameter and dose of Physex. It will also be seen that with a certain dose of Physex, the average egg size is greater when Hexoestrol is given in addition to the Physex dose.

Table 2.

Female silver eels treated with Physex.

No.	Physex I.U. total	Duration of experiment; weeks	Egg diameter mm.
30	2000	11	0.26
105	5500	5	0.27
41	9000	8	0.25
111	11500	13	0.32
108	11500	12	0.35

Table 2 shows the five best results obtained with Physex, only.

In the ovaries of the nine eels shown in Fig. 2 by points

marked 1 we made the very interesting observation that the eggs, which all measured 0.35 mm. or more in diameter, were very loosely connected to each other — an observation which must surely be interpreted as an approaching ripening of the eggs. This observation was made after fixation of the ovaries in 4 per cent formalin. These eggs have thus probably been more nearly mature than in any eel ovary so far observed (except probably the one described by *Calderwood*). The eight experiments in which the eggs exceeded 0.35 mm. in diameter are given in table 3 (Physex + Hexoestrol). The ninth (0.35 mm.) was made with Physex alone (No. 108 in table 2).

Table 3.

Female silver eels treated with Physex and Hexoestrol.

No.	Physex I.U. total	Hexoestrol mg. total	Duration of experiment; weeks	Egg diameter mm.
86	3000	6	12	0.40
83	5000	10	22	0.36
87	5000	10	22	0.41
94	6000	12	22	0.37
91	6000	12	23	0.42
82	7000	14	32	0.41
96	7500	15	36	0.46
90	8500	17	38	0.38

The average egg diameter in these nine eels was more than twice as large as the average egg diameter in untreated silver eels, corresponding to an enlargement by volume of more than 8 times. The eggs of eel No. 96 thus show an enlargement by volume of about 16—17 times. The largest diameter measured in any single egg was 0.5 mm.

Less impressive than the percentage increase by volume, yet more impressive than the percentage increase in diameter, is the percentage increase in area of cross sections. This is evident from Figs. 3 and 4, which show eggs from ovaries of untreated and treated silver eels.

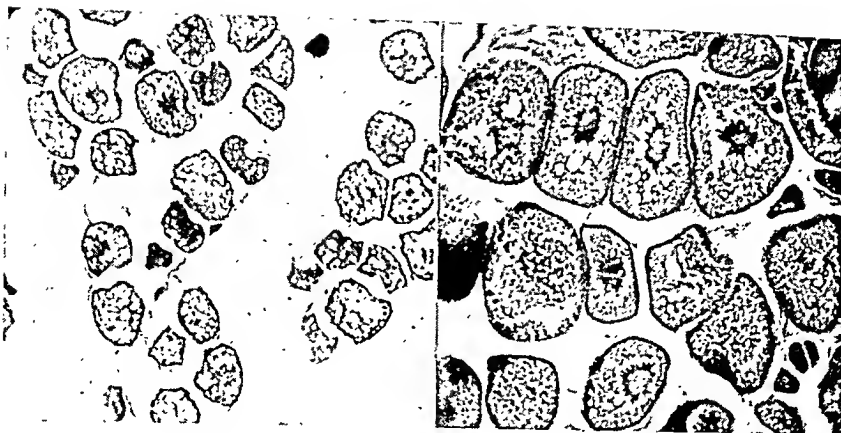


Fig. 3.

(From Naturhistorisk Tidende (1941, p. 90), with permission).
 Section of ovaries, to the left of an untreated female silver eel, to the right of a female treated with Physex + Hexoestrol; average egg diameter 0.161 and 0.375 mm., respectively.

Measurements of the average diameter of eggs of the treated eel of Fig. 3 taken a long time after fixation gave only 0.32 mm., so like also some other eggs they had apparently shrunk somewhat. The figure 0.32 is plotted in Figs. 2 and 5 for this eel to make it more comparable with the other eels. The eggs of this eel were not loosely attached in the ovary. It will be seen from Fig. 3 that the nuclear membranes of the eggs are still visible.

The total length of the silver eels in the control material plotted to the left in Fig. 2 was 49—80 cm. Our silver eels varied from 48—83 cm. The eight experimental eels with eggs exceeding 0.35 mm. in diameter were 58—83 cm. long.

The interval between injections was varied between 3—4 days and 3 weeks. In Fig. 5 the egg diameters are plotted against the average interval between injections, as obtained by dividing the duration of the injection period in weeks by the number of injections given.

It was *a priori* most reasonable to expect more marked effects with small intervals between the doses than with large intervals, and it seems that this expectation is fulfilled in the

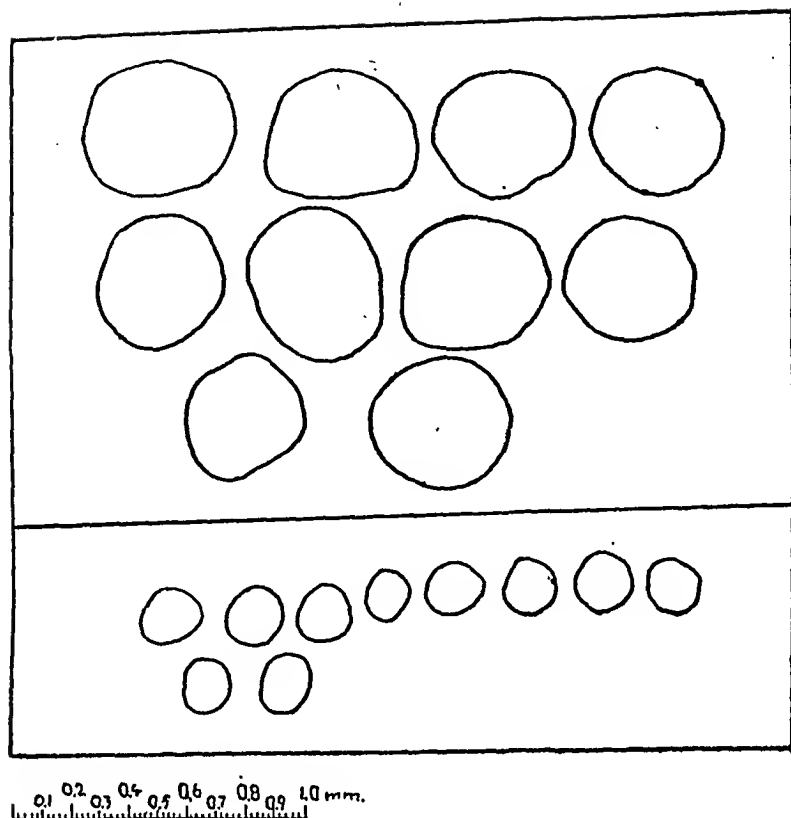


Fig. 4.

Above: eggs of eel No. 96 injected with Physex + Hexoestrol.
 Below: eggs of untreated control eel No. 1.

experiments with Physex alone, though admittedly they are too few for a final conclusion to be drawn. But in the Physex-Hexoestrol experiments longer intervals between the injections appear to bring about more marked effects (i. e. larger eggs). We are inclined to ascribe this to an inhibitory action of Hexoestrol on the pituitary gland. Physex is a chorionic gonadotrophin and has no gonadotrophic activity in hypophysectomized animals. Its effect may therefore be supposed to be aided by a pituitary component (references by *Hamburger*, 1933, and *Burrows*, 1945). There is much to indicate that the internal secretions of the ovary may inhibit the gonadotrophic function of the pituitary (*Moller-Christensen*, 1935). A similar inhibition by Hexoestrol may perhaps explain the

Egg diameter
mm.

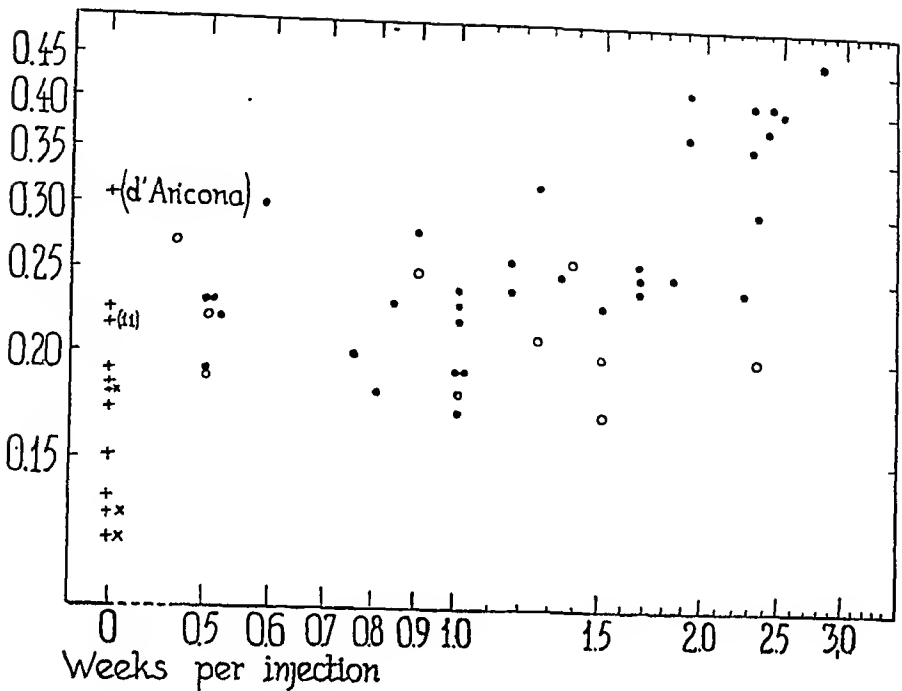


Fig. 5.

Correlation between egg diameter of silver eels and interval between injections of chorionic gonadotrophin (Physex).

+ Controls (from Möbius, 1887; Williamson, 1895; and d'Ancona, 1939).

× Own controls.

• Physex + Hexoestrol (1 mg. per 500 I. U. of Physex).

○ Physex alone.

less marked effects of Physex, the inhibitory action on the pituitary gland of Hexoestrol not having ceased between one injection and the next when the interval is short, whereas it has probably ceased if the interval is made sufficiently long.

Acting on this hypothesis and on the indication of Fig. 2 that large eggs may be obtained with still higher doses, we have planned experiments with still higher doses of Physex given at intervals of three weeks between the injections, and

also injections with Hexoestrol + a non-chorionic gonadotrophic substance known to act in hypophysectomized animals, so that a possible inhibitory action of Hexoestrol on the pituitary gland may be expected to be of no consequence.

ACKNOWLEDGMENTS

We wish to express our sincere thanks to »*Danmarks Akvarium*«, Charlottenlund, Copenhagen, (Director *M. Hojgaard*, M. Sc.) for the facilities offered, and to Dr. scient. *Boje Benzon*, the owner of the firm *Alfred Benzon Ltd.*, for contributing all the preparations of chorionic gonadotrophin (Physex Leo) and synthetic oestrogenic hormone (Hexoestrol A. B.); to Professor, Dr. phil *R. Spärck* and to Dr. phil. *J. Holst Christensen* for the use of *Edinger's* projector; and to Professor, Dr. phil. *Ö. Winge* for photographic assistance.

SUMMARY

Intraperitoneal injections of chorionic gonadotrophin (Physex Leo) into male European silver eels produced full sexual maturity.

The intraperitoneal injection of chorionic gonadotrophin (Physex Leo) into female European silver eels, together with a synthetic non-steroid oestrogen (Hexoestrol A. B.) given intramuscularly, produced growth of the eggs up to a diameter of 0.50 mm. Physex alone was also effective, but the effect was less marked. The largest egg diameter ever measured in non-injected silver eels is 0.31 mm. (*d'Ancona*); usually such eggs measure 0.10—0.25 mm.

Experimentally produced large eggs (exceeding 0.35 mm. in diameter) were loosely attached to the ovary, a sign of approaching maturity.

A positive correlation was observed between hormone dose and effect.

In the Physex-Hexoestrol experiments longer intervals between the injections led to a more marked effect than did the shorter intervals.

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(Professor G. C. Heringa, M. D.)

THE EFFECT OF THE HYPOFUNCTION OF THE
THYROID GLAND, INDUCED BY METHYL-
THIOURACIL, ON THE PHOSPHATASE ACTIVITY
OF SOME ORGANS AND ON THE PROCESS OF
OSSIFICATION IN THE RAT AND IN
THE GUINEA-PIG

BY

D. B. KROON

INTRODUCTION

This paper gives an account of changes in the alkaline- and acid phosphatase activity and of some histological changes in several organs (kidney, liver, spleen, testis, ovary, thyroid gland, adrenal gland, hypophysis) and in the developing skeleton of rats and guinea-pigs, which have been treated with methylthiouracil (antibason, Organon Ltd., Oss.).

The phosphatase activity was not only determined biochemically by the method previously described (*Kroon et al.* 1945, 1948), but the histochemical localization was studied by Gomori's method, modified and improved in our laboratory by *Ruyter & Neumann* (1949). The results obtained by this improved biochemical technique suggest that the blackening of the nuclei, which is attributed by many authors to the nuclear phosphatases, is not due to the ferment reaction, and is only

an artefact. It is probably due to the liberation of phosphate which combines with the calcium ions present in the solution to produce a precipitate of calcium phosphate, in addition to which other insoluble calcium compounds may also be formed. In using the improved technique, the blackening of the nuclei is prevented (the dark colour of the nuclei in the figures of sections treated according to Gomori-Ruyter, noted in this publication, is due to contrast-staining with ten times diluted Ehrlich's haematoxylin).

The results recorded in this paper are part of more extensive experiments on the quantitative relation between a change in the function of an organ and the activity of the phosphomonoesterases. With this object in view we started in the first instance by a consideration of the different metabolic levels which can be induced, partly by the administration of anti-thyroid substances such as methylthiouracil and partly by the administration of thyroxin. This communication will deal exclusively with the effect of the hypofunction of the thyroid gland on the phosphatase activity of some organs and on ossification.

METHODS AND MATERIAL

Rats and guinea-pigs were used as experimental animals. The age of the rats varied from 1—3 months, that of the guinea-pigs from 3—4 months. Experimental and control animals were always of the same sex and were litter-mates.

Methylthiouracil (antibason) was used as the antithyroid substance and was supplied in a pure form by Organon Ltd. The rats received daily doses of 25, 50 or 75 mg.; guinea-pigs a daily dose of 100 mg. The drug was either mixed with the food or suspended in the drinking water with 0.1 per cent agar. The treatment lasted for 2—4 weeks mostly.

In accordance with the results of other investigators we found that the rats reacted very rapidly to antibason treatment with loss of weight. We never observed this in guinea-pigs. This phenomenon is rapidly reversible, for a discon-

tinuation of antibason administration is immediately followed by an increase in weight. It was evident that the temperature at which the rats were kept had a marked effect on this loss of weight.

Rat II^A (fig. 1) which received a daily dose of 50 mg. antibason, was subjected to low room-temperatures, particularly at night. After a few days the body-weight rapidly decreased, while the control-rat II^B, subjected to the same conditions, showed a normal increase in body-weight. Under such conditions the »antibason«-rats usually die within 3 weeks.

If, however, rats treated with antibason are kept at a constant temperature of 30° C no loss of weight occurs and the body-weight remains the same (rat I^A fig. 1) and can be kept at this level for months, by daily doses of methylthiouracil. Rat I^B is the control of rat I^A.

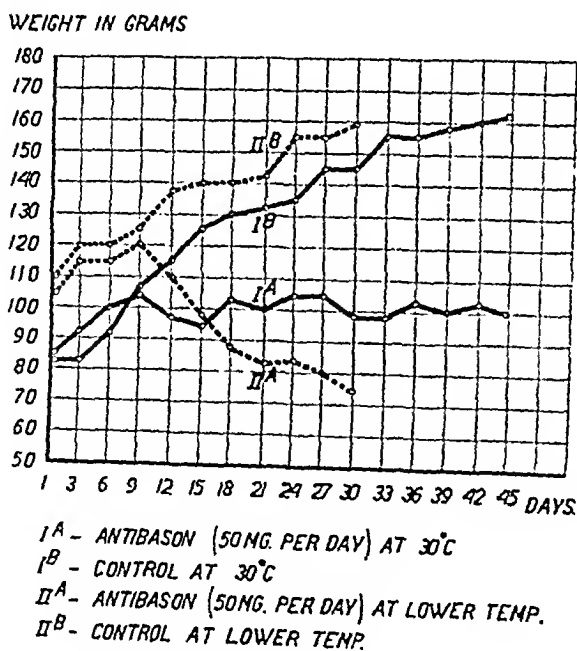


Fig. 1.

Influence of temperature on the body-weight of »antibason«-rats.

Guinea-pigs do not show this susceptibility to low temperatures during the administration of antibason, for even after prolonged treatment with large doses their body-weight in-

creases normally, when the animals are kept at low temperatures. Later, in the discussion on the changes in the pituitary gland, I will return to the effect of temperature.

The biochemical phosphatase determinations were performed by a macro method elaborated in our laboratory; for each determination 0.1 ml. organ-extract was needed (*Kroon et al.*, 1945). For the smaller organs of internal secretion a micro method was used; 0.01 ml. of an organ-extract was sufficient for each determination (*Kroon et al.*, 1948). As far as possible the pH activity curves were determined. For a discussion of their significance I refer to both above mentioned articles.

For histological examination, the tissue was fixed, in the majority of cases, in Pfuhr's mixture without addition of acetic acid; for the histochemical phosphatase localization by the Gomori-Ruyter method we fixed in 80 per cent alcohol. The specimens were embedded in paraffin, and the sections stained with haematoxylin-eosin or with the Mallory-Heidenhain method. The sections of the hypophysis were differentiated with Ruyter's α -Diazot ϵ -staining (*Ruyter*, 1943).

As stated above, Gomori's method, modified by Ruyter and Neumann, was used for the histochemical localization of the alkaline-phosphatase.

RESULTS

Kidneys. As a constant phenomenon a considerable decrease in the alkaline phosphatase activity, in some cases amounting to 50 per cent, was observed in the kidney of animals treated with antibason, while the acid-phosphatase activity remained virtually unchanged. Experiments in vitro have demonstrated that methylthionracil, added in various concentrations to an organ-extract has, as such, no effect on the phosphatase activity (See table 1).

In table 2 the average values of determinations in 13 α -antibason ϵ -rats and in 13 control-rats are recorded. The phosphatase activity is, in this paper, expressed in Bodansky-units.

Table 1 (Kidney).

Phosphatase activity (expressed in mg. liberated P) at various concentrations of antibason in an organ extract.

Concentration of antibason in the reaction mixture, mg./ml.	Phosphomonoesterase I	Phosphomonoesterase II
	liberated P (in mg.)	liberated P (in mg.)
0	1.94	1.02
0.01	1.82	0.99
0.1	1.82	0.96
0.5	1.84	0.96
1.5	1.94	0.90
3	2.12*)	0.89

*) At this very high concentration, which is never attained in vivo, a small activation occurs.

Table 2 (Kidney).

Acid and alkaline phosphatase activity of the kidney of »antibason«-rats and control rats.

Control-rats		"Antibason"-rats	
pH 5.4 459 B.U.	pH 10.0 2739 B.U.	pH 5.4 455 B.U.	pH 10.0 1471 B.U.

Table 3 (Kidney).

Acid and alkaline phosphatase activity of the kidney of »antibason«-guinea-pigs and control guinea-pigs.

Control-guinea-pigs		"Antibason"-guinea-pigs	
pH 5.4 100 B.U.	pH 10.0 1627 B.U.	pH 5.4 105 B.U.	pH 10.0 945 B.U.

The decrease in the alkaline phosphatase activity of the kidney of the guinea-pig was in all cases less marked than in that of the rats. It should be pointed out that the normal ac-

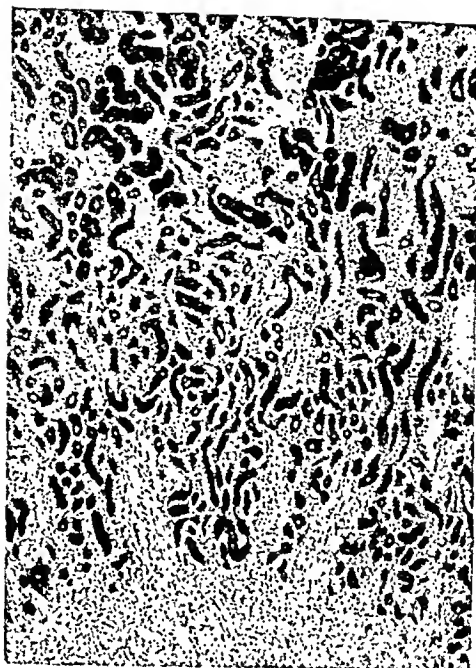


Fig. 2.

Kidney of hypothyroid rat; reduced phosphatase activity especially in the partes rectae of the portiones principales. Technique: Gomori-Ruyter; nuclear stain: 10 \times diluted Ehrlich's haematoxylin. 86 \times .

tivity is also less in guinea-pigs. In table 3 average values of determinations in three experimental and in three control-animals are recorded.

The histochemical sections according to Gomori-Ruyter, which were made exclusively from the kidney of the rat, proved that the decreased phosphatase activity was localized especially in the partes rectae of the portiones principales of Henle's loops. The parts clearly showed a less pronounced blackening than did the corresponding parts of the control kidney (figs. 2 and 3), while in the proximal convoluted tubules of the renal cortex practically no differences could be seen. I never observed any changes in the histological structure of the kidney of »antibason«-animals.

Liver and spleen. The administration of antibason in rats is followed by an increased alkaline phosphatase activity in both these organs. This is more pronounced in the liver than

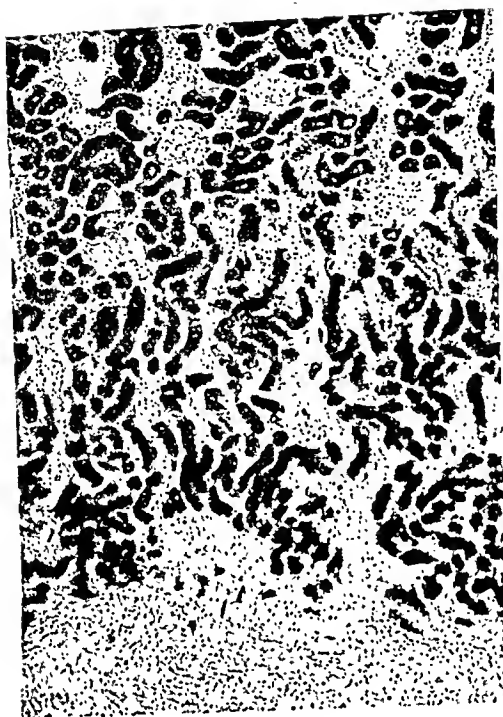


Fig. 3.

Kidney of normal control rat. Technique: Gomori-Ruyter; nuclear stain: see figure 2. 86 X.

in the spleen. The acid phosphatase activity diminishes somewhat in the liver, and does not change at all, or only very slightly, in the spleen. Antibason did not induce changes in guinea-pigs. See table 4 for the liver of the rat (average values from 10 determinations) and table 5 for the liver of the guinea-pig (average values from 3 determinations).

As I performed a less extensive investigation of the spleen I have not sufficient data for this organ at my disposal.

Table 4 (Liver).

Acid and alkaline phosphatase activity of the liver of »antibason«-rats and control rats.

Control-rats		"Antibason"-rats	
pH 5.4 392 B.U.	pH 10.0 36 B.U.	pH 5.4 337 B.U.	pH 10.0 66.3 B.U.

Table 5 (Liver).

Acid and alkaline phosphatase activity of the liver of »antibason«-guinea-pigs and control guinea-pigs.

Control-guinea-pigs		"Antibason"-guinea-pigs	
pH 5.4	pH 10.0	pH 5.4	pH 10.0
152 B.U.	21 B.U.	148 B.U.	19 B.U.

The liver had our special attention as a similar change in the phosphatase activity (an increase of the alkaline and a decrease of the acid) of the same percentage magnitude was observed in our laboratory by *Neumann* (1948) in mice, which had been kept on a diet with a low carbohydrate and a high fat content. The question whether the liver metabolism in hypothyroid animals undergoes similar changes as in these dielectric experiments will be further investigated.

Neumann (1948) suggests that the increased alkaline-phosphatase activity in his experiments »might play a part in the synthesis of glycogen from other substances than glucose«. In this connection it is interesting to note that at the histochemical localization of glycogen (by Best's method) in the liver of a hypothyroid rat, which had lost considerably in weight, it appeared that this liver contained a fairly large quantity of glycogen. This glycogen was especially localized around the central veins. The liver of the control-rat also contained glycogen, which was, however, more diffusely distributed. Histological changes in the liver were never seen in »antibason«-animals.

Testis and ovary. Widely divergent and often contradictory results were obtained in the phosphatase determinations. The dispersion of the phosphatase activity was already fairly large in the control-animals used for these experiments. For the acid phosphatase it varied from 105—340 B. U., for the alkaline phosphatase activity from 135—280 B. U. In the hypothyroid animals the histological picture of the testis shows in some cases a total atrophy of the semenproducing epithe-

lium as well as of the connective tissue; in other cases there was a partial atrophy, while in some animals spermatogenesis was absolutely normal. In most cases of atrophy the activity, especially of the alkaline phosphatase, had diminished, although in some cases it was within normal limits. In the ovaries of hypothyroid animals, the phosphatase activity showed differences in some cases, while in other cases no alterations occurred. Only extensive statistical investigations taking into account age, function, generation etc. will make it possible to obtain an insight into this problem. This is certainly important, for it is just these marked physiological variations which make these organs such suitable material for the study of the relation between functional condition and phosphatase activity.

Thyroid gland. After administration of methylthiouracil the thyroid gland increases considerably in size and becomes hyperaemic. The average weight of the normal thyroid of control-rats was 12.5 mg.; in the »antibason«-rats this weight was 5—6 times as great. In the guinea-pig the average normal weight was 66.6 mg., while the »antibason« thyroid gland had a weight of 260 mg. The ratio between dry-weight and wet-weight (determined in 6 normal thyroid glands of rats) was 5.5. The same ratio determined in 6 »antibason« thyroid glands was 4.3. Hence the dry substance definitely increases in these latter animals. The organs were weighed, one after the other, on a microbalance. Drying was performed by the »freezing-drying« method. As a consequence mitoses could always be seen in the sections (fig. 4; fig. 5 is of a normal thyroid gland). The »antibason« thyroid shows the histological picture of a hyper-active thyroid gland. This apparently paradoxical phenomenon has been explained by the experiments of Astwood and others, which proved that thiouracil blocks the thyroxin production. This results in a thyroxin deficiency in the organism, stimulating the pituitary gland to increased thyrotrophic function. This in turn brings about a hyperactive state of the thyroid which is represented in the histological picture by



Fig. 4.

Hyperactive thyroid gland of an »antibason«-rat (at first 25 mg.; later on 50 mg. for 23 days); 3 mitoses. Technique: Pfuhl-Duazor; 460 \times .

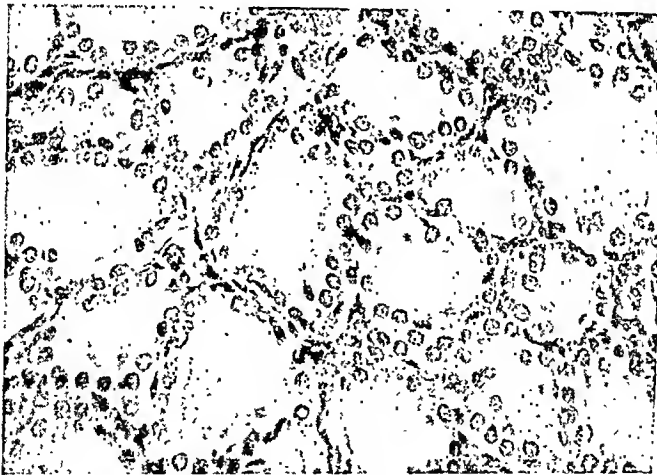


Fig. 5.

Normal thyroid gland of a control rat. Technique: Pfuhl-Haem.-Eos. 460 \times .

follicles with high cells and containing practically no colloid.

In the phosphatase activity of the »antibason« thyroid gland, which was determined by the micro method, an increase was found on the alkaline side, while on the acid side

the change was less marked. In table 6 the average values of determinations made in 7 rats are recorded.

Table 6 (Thyroid).

Acid and alkaline phosphatase activity of the thyroid of »antibason«-rats and control rats.

Control-rats		"Antibason"-rats	
pH 5.4 66 B.U.	pH 10.0 176 B.U.	pH 5.4 74 B.U.	pH 10.0 220 B.U.

In guinea-pigs we found no distinct differences between the phosphatase activity in the hyperactive thyroid gland and of the normal glands. Table 7 gives the average figures of 4 »antibason«- and 4 normal guinea-pigs.

In the normal guinea-pig the activity of the acid phosphatase is greater than in the rat, while that of the alkaline phosphatase is lower.

Table 7 (Thyroid).

Acid and alkaline phosphatase activity of the thyroid of »antibason«-guinea-pigs and control guinea-pigs.

Control-guinea-pigs		"Antibason"-guinea-pigs	
pH 5.4 178 B.U.	pH 10.0 90 B.U.	pH 5.4 190 B.U.	pH 10.0 83 B.U.

Interesting is the histochemical localization of the phosphatase in the hyperactive »antibason« thyroid gland. In the normal thyroid of the rat the phosphatase is found exclusively in the endothelium of a few scattered capillaries. In the hyperactive thyroid gland all endothelia of the distended blood-vessels have become phosphatase-positive (figs. 6, 7, and 8). It can obviously be assumed that the increased phosphatase activity in the endothelium is related to the increased secretion of colloid into the blood. This question will be further investigated since the phosphatase is exclusively found in the capil-

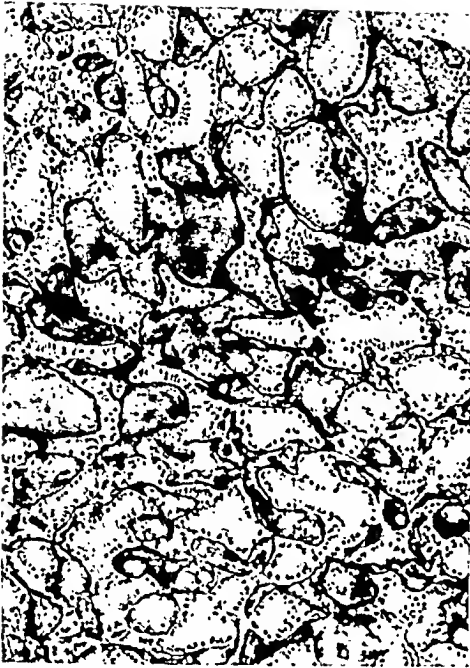
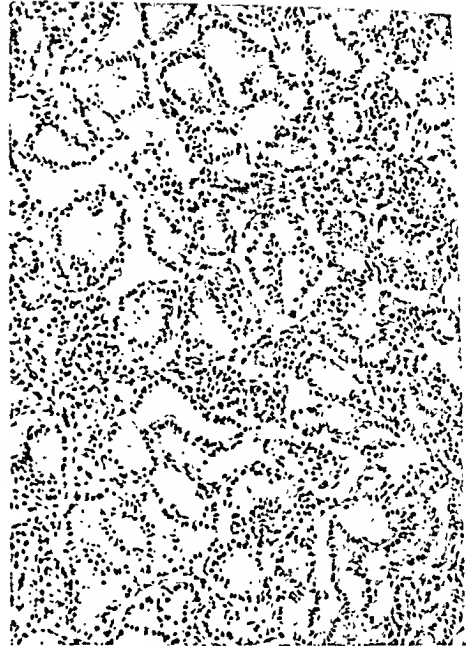
*Fig. 6.**Fig. 7.**Fig. 8.*

Fig. 6.

»Antibason« thyroid gland of a rat (50 mg. antibason daily for 1 month) according to Gomori-Ruyter. All endothelial cells of the wide bloodvessels are phosphatase-positive. Nuclear stain: see figure 2. 150 X.

Fig. 7.

Control section of the same »antibason« thyroid. Technique: Gomori-Ruyter, using calcium nitrate instead of β -calcium-glycero-phosphate. Nuclear stain: see figure 2. 150 X.

Fig. 8.

Gomori-Ruyter section of the thyroid of a control rat. The endothelial cells are phosphatase-positive in certain places only. Nuclear stain: see figure 2. 150 X.

lary endothelium in other endocrine organs, too (as will be seen below). In some cases this localization is limited to definite parts of the gland.

Pituitary gland. In the »antibason«-rats the weight of the pituitary gland had decreased to ± 50 per cent. In control-rats the weight of the pituitary gland was ± 12 mg., in the »antibason«-rats ± 6 mg. In a few of my determinations I found that the alkaline phosphatase in the »antibason« pituitary gland was slightly increased (normal 180—200 B. U. at pH 10.0). No phosphatase determinations were made in the hypophysis of the guinea-pig. As the hypophysis of »antibason«-rats was very small, I had not enough organ-extract at my disposal for the determination of the acid phosphatase. In the histological examination we found considerable alterations in the anterior lobe of the pituitary of »antibason«-rats. The α -cells are either absent, or their number is diminished and the few which are still present have become small cells containing a few red granules, surrounding the nucleus. The β -cells, on the other hand, show a marked activation, characterized by large vacuolized hypochromatic elements, whereas hyperchro-



Fig. 9.

Hypophysis of a rat treated with antibason (50 mg. daily for 1 month). Activated β -cells, no α -cells. Technique: Pfuhl-Duazor. 460 \times .

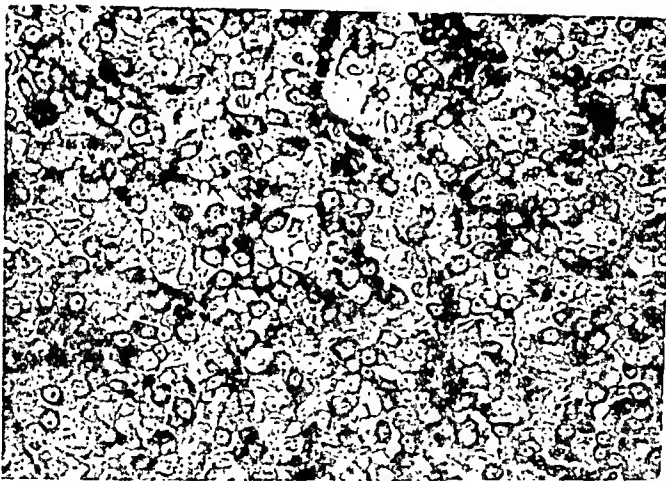


Fig. 10.

Normal hypophysis of a control rat. Technique: Pfuhl-Duazor. 460 \times .

matic forms with a pyknotic nucleus also occur (figs. 9 and 10). This supports the opinion, held by many authors, that the thyrotrophic function of the hypophysis is associated with the β -cells, as we already know that the pituitary of »antibason«-rats has an increased thyrotrophic function. In the »antibason«-guinea-pig I never found this appearance in the hypophysis.

The α -cells do not alter and the β -cells do not show activation. In this connection it is important to note that the hypophysis of the guinea-pig has only a very small thyrotrophic activity (Loeb, 1932, and *Brolin*, 1945). It can thus be understood that this gland will react only very slightly when the thyroxin-content of the blood is decreased. The disappearance in the rat of the acidophilic cells under the influence of antibason and the fact that these cells do not disappear in the guinea-pig, agrees with the finding that there is a disturbance in the growth of the rat's skeleton but not in that of the guinea-pig. That these two facts are related to each other is made very probable by the character of the histological changes in the developing skeleton of the rat (these changes will be discussed in the following part of this paper, while the histological picture of the developing skeleton in the »antibason«-guinea-pig remains absolutely normal.

Here I wish to refer to a publication by *Brolin* (1945) on the structural and hormonal reactions found in the anterior lobe of the hypophysis of rats which have been subjected to low temperatures. The regulatory effects of the anterior lobe of the hypophysis were particularly studied.

The physiological mechanism is similar to that which occurs when methylthiouracil is administered: due to cooling, the thyroxin demand of the organism is increased and this stimulates the hypophysis to an increased thyrotrophic function. The histological alterations, which *Brolin* observed in his rats exposed to cold, have an astonishing similarity to the findings with antibason: identical pictures of hyperactivity were found in the thyroid and identical changes of the β -cells into activated hypochromatic and vacuolized elements. Only the acidophilic cells remain unaltered in rats exposed to cold. Consequently *Brolin* did not find any disorder in growth. These experiments too proved that the rat is very sensitive to a thyroxin-deficiency. It is easily understood that under the influence of the combination of two identically directed factors, viz. antibason and cold, the rats react more strongly than under the influence of antibason only.



Fig. 11.

Gomori-Ruyter section of the hypophysis of an »antibason«-rat. Only the endothelia of the bloodvessels in the posterior lobe are phosphatase-positive. Nuclear stain: see figure 2. 150 X.

The histochemical examination proved that, in the pituitary the phosphatase is localized exclusively in the endothelial cells of the blood-vessels of the posterior lobe. Cells and blood-vessels of the anterior lobe are always phosphatase-negative. As I found no difference in phosphatase localization in the normal hypophyses and in the »antibason« hypophyses, I only show a picture of the pituitary of an »antibason«-rat (fig. 11).

Adrenal gland. The adrenals of hypothyroid rats had decreased in weight (± 12 mg. for the »antibason«-rats and ± 24 mg. for the normal rats). In a few of these adrenals the phosphatase activity was determined. We found that the alkaline phosphatase activity had increased somewhat, whereas the acid phosphatase activity remained unchanged as compared with the control-adrenals; e. g. in the control-rat: pH 4.4: 230 B. U.; pH 10.2: 470 B. U.; in the »antibason«-rat: pH 4.4: 220 B. U.; pH 10.2: 705 B. U. I performed only a few determinations in the guinea-pig; the alkaline phosphatase activity showed a slight increase, the acid phosphatase activity remained unchanged: control adrenal: pH 4.4: 40 B. U.; pH



Fig. 12.

Gomori-Ruyter section of the adrenal gland of an »antibason«-rat (50 mg. daily during 1 month). Nuclear stain: see figure 2. 86 X. Phosphatase-positive capillaries in the cortex and some in the medulla.

10.2: 2280 B. U.; »antibason« adrenal: pH 4.4: 40 B. U.; pH 10.2: 3000 B. U.

In a few cases I found enlarged adrenals in the guinea-pig. Although other authors found hypertrophy of the rat's adrenal cortex following the administration of methylthiouracil, I did not find any essential histological alterations in my »antibason«-rats.

The preparations according to Gomori-Ruyter showed that when antibason had been administered the capillaries of the cortex were far more extensively phosphatase-positive than in the control animals. This could, as in the »antibason« thyroid, point towards an increased action on the part of the capillaries endothelium in secretion (figs. 12 and 13). It is very striking that this should be especially found in those capillaries which



Fig. 13.

Gomori-Ruyter section of the adrenal gland of a control rat. Nuclear stain: see figure 2. 150 X. Only a few capillaries are phosphatase-positive; the majority are localized in the zona glomerulosa.

are situated more peripherally in the cortex. The wide sinusoids in the medulla are always phosphatase-negative. Although other adrenals still have to be studied, the above-mentioned facts are supported by *Neumann's* findings in the adrenals of a cow. Here the adrenal cortex and medulla are easily separated, and the alkaline phosphatase was almost exclusively present in the cortex.

The growing skeleton. Cessation of growth of the skeleton is one of the most striking features of hypothyroid rats. By administering thiouracil to rats, from birth onwards, *Hughes* (1944) obtained characteristic cretins. When the phosphatase activity is determined in the skeleton (e. g. in a femur, radius and ulna, or a rib) it appears that in cases where antibason has been administered, the alkaline phosphatase activity decreased considerably (mostly ± 25 per cent), whereas the acid phosphatase activity showed no changes. In guinea-pigs we found that the alkaline phosphatase had diminished somewhat in all cases, although no disorders of growth occur in these animals. Table 8 (average values of 5 determinations in the

rat) and table 9 (average values of 3 determinations in the guinea-pig):

Table 8 (Femur).

Acid and alkaline phosphatase activity of the femur of »antibason«-rats and control rats.

Control-rats		"Antibason"-rats	
pH 5.4 50 B.U.	pH 10.0 752 B.U.	pH 5.4 45 B.U.	pH 10.0 453 B.U.

Table 9 (Femur).

Acid and alkaline phosphatase activity of the femur of »antibason«-guinea-pigs and control guinea-pigs.

Control-guinea-pigs		"Antibason"-guinea-pigs	
pH 5.4 40 B.U.	pH 10.0 308 B.U.	pH 5.4 38 B.U.	pH 10.0 246 B.U.

The histological pictures of the zone of growth in the skeleton of hypothyroid rats, which had stopped growing, were very interesting. A total disappearance of the spongiosa bars and a reduction in the hypertrophic zone of cartilage are seen. This latter zone is separated from the diaphysal marrow-cavity by a transverse bone plate (figs. 14, 15, 16, 17). These pictures are absolutely identical with those seen in hypophysectomized rats. (*Freud et al.*, 1939).

In cases of cretinism in man the skeleton shows alterations which are identical with those found in pituitary dwarfs (*Weinmann & Sicher*, 1947). In a publication by *Todd, Wharton & Todd* (1938), on the influence of thyroid deficiency on the growth of the skeleton of sheep, the real cause of the retarded growth of the skeleton is attributed to the anterior lobe of the hypophysis and not to decreased basal metabolism as such.

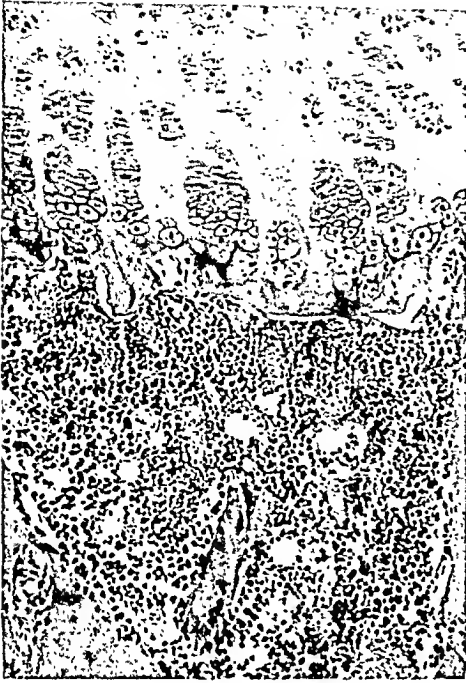
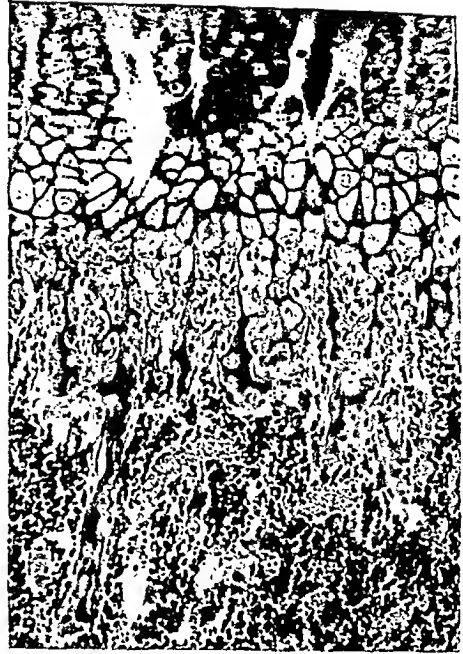
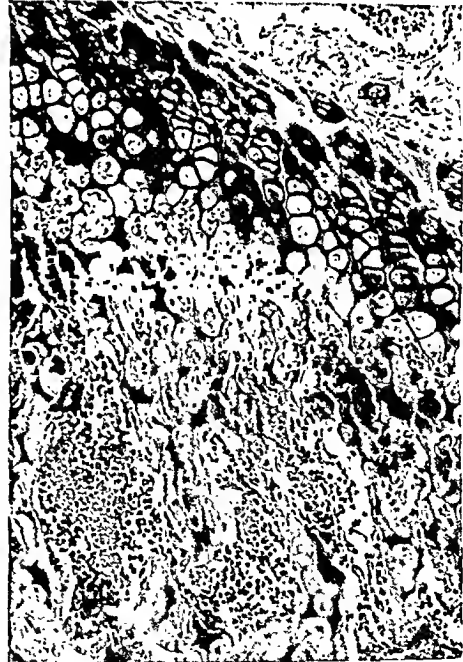
*Fig. 14.**Fig. 15.**Fig. 16.**Fig. 17.*

Fig. 14.

Transverse bone plate (rib) in an »antibason« rat (after 18 days, at first 50 mg. daily, later on 25 mg.). Age 2 months. Technique: Pfuhl-Haem.-Eosin. 175 X.

Fig. 15.

Epiphyseal cartilage plate of the rib of a control rat. Technique: Pfuhl-Haem.-Eosin. 175 X.

Fig. 16.

Transverse bone plate against the epiphyseal cartilage plate of the femur of an »antibason«-rat (same rat as in figure 14). Technique: Pfuhl-Haem.-Eosin. 175 X.

Fig. 17.

Epiphyseal cartilage plate of the femur of a control rat. Technique: Pfuhl-Haem.-Eosin. 175 X.

SUMMARY AND CONCLUSION

1. In cases of a hypofunction of the thyroid induced by methylthiouracil (antibason), a change in the phosphatase activity of the organs of rats and guinea-pigs is observed; this change involves only the alkaline phosphatase, whereas the acid phosphatase shows no change, or only a slight one.

2. In hypothyroid rats and guinea-pigs a decrease in the alkaline phosphatase activity is found in the kidney and in the developing skeleton. This decrease is particularly localized to the partes rectae of the portiones principales of the renal tubules and to the brushborder.

3. An increase in the alkaline phosphatase activity occurred in the liver and spleen of hypothyroid rats. In the liver of the rat this increase was accompanied by a diminution of the acid phosphatase activity.

4. In hypothyroid rats an increase in the alkaline phosphatase activity was found in the thyroid gland, which showed consistently a clear picture of activation following antibason administration; this was seen in the hypophysis, where the α -

cells disappeared and the β -cells changed into large activated cells, and in the adrenal gland where no marked histological changes were seen. The histochemical examination proved that in these three endocrine glands the endothelium of the bloodvessels had become phosphatase-positive to a greater extent than in normal control glands.

5. In hypothyroid rats we observed a reduced growth of the skeleton.

6. Widely varying results were obtained when phosphatase determinations were performed in testes and ovaries of hypothyroid animals.

I should like to express my gratitude to the Rockefeller-Foundation for its contribution which enabled me to carry out this investigation.

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CHANGES IN THE ASCORBIC ACID CONTENT IN THE INTERSTITIAL GLAND OF THE RABBIT OVARY FOLLOWING GONADOTROPHIC STIMULATION¹⁾

BY

LENNART CLAESSION, NILS-ÅKE HILLARP, BERTIL HÜGBERG
and BERNT HÖKFELT

Long, Sayers, and co-workers (Sayers, Sayers, Lewis & Long, 1944, Sayers, Sayers, Liang & Long, 1945, 1946, Long, 1947) have demonstrated in a series of investigations that the adrenocorticotrophic hormone causes a marked and rapid decrease in the content of ascorbic acid in the adrenal glands. This reaction appears to be so specific that it can be used as a method of assay for this hormone (Sayers, Sayers & Woodbury, 1948). Other steroid-forming cell systems have not been investigated in this respect. Such an investigation seems desirable, however, for the proper evaluation of the role of ascorbic acid in steroid synthesis. The changes in the ascorbic acid content of the interstitial gland of the ovary of rabbits following gonadotrophic stimulation have, therefore, been studied.

¹⁾ Aided by a grant from Statens Medicinska Forskningsråd.

MATERIAL AND METHODS

Pregnant rabbits only were used in the experiments (12th day of pregnancy) as the ovaries of pregnant rabbits are almost entirely composed of interstitial gland — except the corpora lutea which are easily removed. The interstitial cells, moreover, are relatively inactive during pregnancy in comparison with the highly active state of the cells after administration of 450 I. U. of pregnant mare serum gonadotrophin.

On the 12th day of pregnancy one ovary was removed (sodium N-methyleylelohexenylmethylbarbiturate anaesthesia) and at the same time 450 I. U. of pregnant mare serum gonadotrophin (Antex Leo¹) was injected intravenously. The remaining ovary was removed after 45 min., 1½, 3, 6, 12, 24, and 48 hours respectively. Immediately after the removal of the ovaries the corpora lutea were removed; all large follicles were punctured, and the ovaries were weighed. The ovaries were ground with sand in a mortar and extracted with 4.5 per cent trichloroacetic acid. The extracts were then treated with norit following which an immediate determination of the ascorbic acid content was carried out after coupling with 2,4-dinitrophenylhydrazine, according to *Roe, Kuether*, and co-workers (*Roe & Kuether*, 1943, *Roe & Oesterling*, 1944, *Mills & Roe*, 1947). The colour was measured by means of a Spekker photo-electric colorimeter 30 minutes at the earliest after addition of H_2SO_4 .

The animals were kept on a diet of turnips, hay, and corn.

RESULTS

The calculation of the changes in the ascorbic acid content is complicated by the fact that the ovaries on gonadotrophic stimulation rapidly increase in weight (see Table 1 and Fig. 1). As has been pointed out in a previous paper (*Claesson, Diszfalusy, Hillarp & Högberg*, 1948), this increase in weight is largely due to hyperaemia among other causes. Even as

¹) Leo and Co., Hälsingborg, have kindly placed this preparation at our disposal.

long as 24 hours after the injection, increase in the number of cells can represent not more than 50 per cent of the increase in the wet weight. It would thus be incorrect simply to state the ascorbic acid content per gm. of ovarian tissue after the administration of PMS.

For the evaluation of the changes in the ascorbic acid content, the increased weight of the ovaries has been studied in a large number of pregnant rabbits (Table 1). One ovary was removed on injection of 450 I. U. of PMS, the other at different times after the injection: the ovaries were weighed without the corpora lutea. The increase in weight has been calculated in column I as the average of the increases in weight for the individual animals. Since it is possible, however, that there is a correlation between the weight of the ovaries *before*

Table 1.

Increase in weight in the ovaries of pregnant rabbits (6—12th day of pregnancy) following gonadotrophic stimulation. One ovary was removed at the time of the injection of 450 I. U. of PMS intravenously, the other 1½—48 hours after the injection. The corpora lutea were removed. In column I the increase in weight is given as the average of the increases in weight in the ovaries of the individual animals, in column II the increase in weight is calculated on the total weight of the ovaries before and after stimulation.

No. of animals	Time in hours	Total weight of ovaries		Increase in weight	
		before stimulation mg.	after stimulation mg.	I per cent	II per cent
20	1½	6627	6933	3.7	4.6
16	3	6200	6408	2.0	3.4
17	6	6428	8545	34	33
15	12	4558	7303	61	60
22	24	7488	11454	52	53
7	48	1800	2455	41	36

the stimulation and their change in weight *after*, the increase in weight calculated on the total weight of the ovaries before and after the stimulation is given in column II. It appears from the table, however, that both these methods of calculation give the same result.

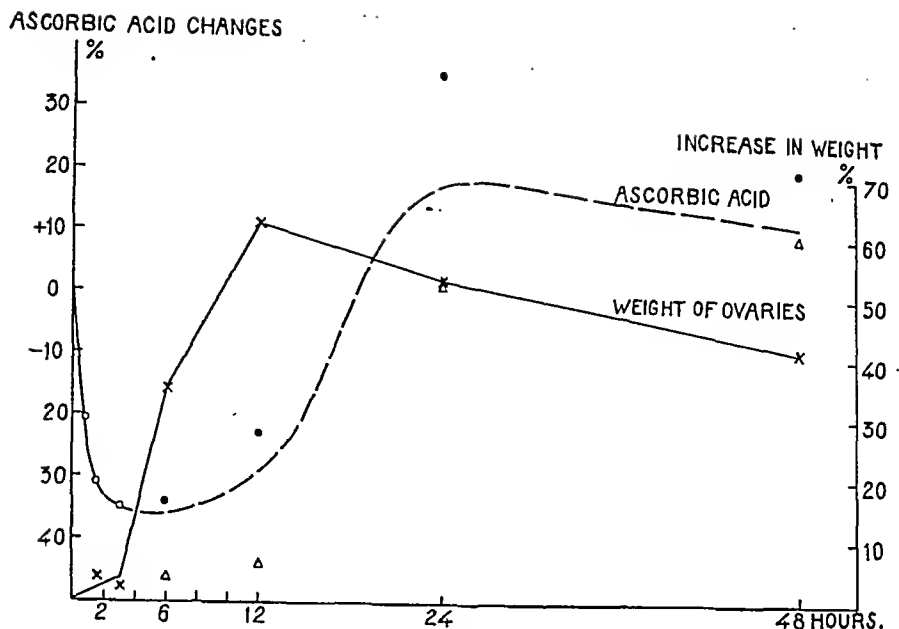


Fig. 1.

Changes in the ovarian weight and in the ascorbic acid content of the interstitial gland on gonadotrophic stimulation. The ovaries are removed at various intervals after an intravenous injection of 450 I. U. of PMS. The broken line indicates the probable curve of the ascorbic acid changes later than 3 hours after the injection (see the text).

● = values calculated according to II in table 2.

△ = " " " " III " " "

It is evident from the weight curve in Fig. 1 that the weight of the ovaries remains practically unchanged for almost 3 hours. After this period there is a marked and rapid increase in weight. It is, therefore, possible to state the ascorbic acid content per gm. of ovarian tissue during the first three hours following the PMS-injection without introducing any error. Later on it is possible to state only the limits within which the correct values are to be found. As in a previous study in which the calculation of the lipid changes in the ovary was carried out (see *Claesson, Diszfalusy, Hillarp & Högberg, 1948*), the ascorbic acid content is given in the present report partly on the basis of the weight of the ovary first removed

(column II in Table 2), and partly on the basis of a weight calculated on the assumption that half the increase in weight is caused by an increase in the number of cells (column III). The first method of calculation gives the upper limit and the second the lower limit of the values of the ascorbic acid content. For the period between 6—12 hours after the injection of PMS, the real values will probably lie nearer the upper limit than the lower, since no considerable increase in the number of cells in the interstitial gland can have occurred at such an early stage. It is more likely that hyperaemia, increased tissue fluid, etc., play the most important role in the increase in weight.

It appears from Table 2 that the interstitial gland has a relatively high ascorbic acid content (average = 0.70 ± 0.14 mg./gm.). As early as 45 minutes after the injection of 450 I. U. of PMS the ascorbic acid content was considerably lowered (average = 21 per cent). This decrease continued rapidly for another 45 minutes (average = 31 per cent) after which it occurred much more slowly (average = 35 per cent at 3 hours). After this period it is no longer possible to register exactly the course of the changes, but, taking into consideration the analysis of the increase in weight of the ovaries presented above, it seems very probable that changes in the ascorbic acid content can be represented by the broken curve shown in Fig. 1. According to this curve, the decrease reaches a maximum of 35 to 40 per cent between 3 and 6 hours. Afterwards a slow increase occurs in the ascorbic acid content which reaches normal or even higher values 24 and 48 hours after the injection. — It seems also evident from the course of the curve between 0 and 3 hours that the curve between 3 and 12 hours must, in fact, lie nearer the upper limit values.

The content of ascorbic acid in the blood is so low (average value for the animals used = 1.3 mg./100 ml.) that even a strong hyperaemia cannot influence the ascorbic acid content of the ovary to any appreciable degree.

Before stimulation		I	After stimulation				
Weight of ovaries	Ascorbic acid content		Weight of ovaries	Ascorbic acid content	Change in ascorbic acid content per cent		
mg.	mg./gm.		mg.	mg./gm.			
189	0.71	45 min.	180	0.58	-18		
286	0.85	» »	293	0.72	-15		
293	0.74	» »	263	0.65	-12		
335	0.54	» »	412	0.35	-35		
455	0.80	» »	580	0.56	-30		
207	0.65	» »	210	0.55	-15		
289	0.51	11½ hrs.	352	0.33	-35		
187	0.62	» »	176	0.48	-23		
577	0.35	» »	619	0.25	-29		
355	1.02	» »	356	0.63	-38		
261	0.73	» »	303	0.51	-30		
161	0.64	3 hrs.	185	0.48	-25		
326	0.45	» »	374	0.29	-36		
146	0.86	» »	191	0.49	-43		
665	0.65	» »	681	0.41	-37		
				II	III	II	III
229	0.71	6 hrs.	320	0.43	0.36	-39	-49
176	0.65	» »	227	0.48	0.37	-26	-43
92	0.87	» »	141	0.55	0.36	-37	-47
243	0.87	12 hrs.	504	0.67	0.44	-23	-49
215	0.70	» »	377	0.46	0.40	-34	-43
199	1.10	» »	328	0.93	0.70	-15	-36
123	0.73	» »	268	0.69	0.45	-5	-38
201	0.87	» »	377	0.47	0.33	-47	-62
297	0.84	» »	546	0.74	0.52	-12	-38
211	0.69	24 hrs.	236	0.68	0.64	-1	-7
346	0.68	» »	526	0.81	0.54	+19	-21
130	0.58	» »	230	0.62	0.44	+7	-24
120	0.75	» »	200	1.08	0.65	+44	-13
174	0.66	» »	300	1.55	1.14	+135	+73
540	0.64	» »	583	0.71	0.68	+11	+6
249	0.66	48 hrs.	319	0.82	0.71	+24	+8
172	0.63	» »	198	0.70	0.65	+11	+3
260	0.73	» »	284	0.77	0.74	+5	+1
219	0.57	» »	336	0.85	0.67	+53	+18
197	0.63	» »	268	0.94	0.79	+49	+25
246	0.57	» »	180	0.42	0.57	-26	0
111	0.59	» »	166	0.90	0.72	+33	+22
331	0.57	» »	463	0.68	0.57	+19	0

Table 2.

Changes in the ascorbic acid content of the interstitial gland (rabbit ovaries, 12th day of pregnancy) on gonadotrophic stimulation. The first ovary was removed immediately before an intravenous injection of 450 I. U. of PMS, the second was removed at varying intervals after the injection (I). — The ascorbic acid content of the stimulated ovaries which were removed later than 3 hours after the injection is calculated on the wet weight of the ovary first removed (II) as well as on its own wet weight reduced as described by *Claesson, Diszfa-lusy, Hillarp & Högberg, 1948* (III).

DISCUSSION

Following gonadotrophic stimulation there is a rapid and marked reduction in the ascorbic acid content of the interstitial gland of the ovary similar to that found in the adrenal gland on corticotrophic stimulation (*Long, Sayers, and co-workers*). The curve of the ascorbic acid changes in the rabbit ovary follows approximately the same course as that of the adrenal glands of guinea-pigs (*Sayers, Sayers, Liang & Long, 1946*). It seems somewhat premature, however, to draw any conclusions whatever concerning the role played by ascorbic acid in steroid synthesis in these organs. It is true that the ascorbic acid content is high in the steroid producing cell systems, but other endocrine organs, such as the pituitary gland (pars anterior and intermedia) and the adrenal medulla, have a high ascorbic acid content (for references see *Giroud, 1938*); furthermore a marked reduction in the ascorbic acid content of the anterior lobe of the pituitary gland after increased hormone secretion has also been observed (*Pincus & Berkman, 1947*). The question may, therefore, be raised as to whether the ascorbic acid is involved in the general metabolism of the cells.

It is particularly interesting to note that the changes in the ascorbic acid content occur so rapidly after the administration of PMS. The main decrease takes place during a period when no changes in the lipid content of the interstitial gland can as yet be demonstrated (unpublished results).

SUMMARY

The changes in the ascorbic acid content of the interstitial gland of the ovary were studied in pregnant rabbits (12th day of pregnancy) at different periods of time (45 minutes — 48 hours) after an intravenous injection of 450 I. U. of pregnant mare serum gonadotrophin. The gonadotrophic stimulation produces a marked and rapid reduction in the ascorbic acid content (21 per cent after 45 min., 31 per cent after 1½ hour and 35 per cent after 3 hours), probably reaching a maximum between 3 and 6 hours after the injection. Later there is a slow increase in the ascorbic acid content to normal or higher values after 24 and 48 hours.

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TESTOSTERONE TREATMENT AND 17-KETOSTEROID EXCRETION

INVESTIGATIONS ON THE INFLUENCE OF THE MODE
OF ADMINISTRATION UPON THE ABSORPTION AND
EXCRETION OF TESTOSTERONE PROPIONATE*)

BY

CHRISTIAN HAMBURGER and SIGVARD KAAE

The production and release of the hormones from the endocrine glands are regulated according to the immediate demand of the organism by the nervous system and by the finely balanced interrelationship existing between these glands. In normal conditions, the secretion is assumed to be a fairly continuous process.

When the sexual and adrenal cortical hormones are used therapeutically in order to substitute a decreased production, the most commonly used parenteral mode of administration has consisted of intramuscular injections of oily solutions, given twice or three times weekly. This method of administering the hormones must, however, be regarded as rather unphysiological, as the concentration of the substances in the circulating blood will necessarily change from excessively high

*) Paper read at the meeting of the Danish Society for Endocrinology May 2, 1949.

to very low values. The utilization of the hormones has been shown to be rather incomplete when given in this way. In the last decade much work has been done in order to elaborate more economical methods of administration.

A more continuous absorption, and hence a more uniform level of the particular hormone in the blood, could be obtained by shortening the intervals between the injections, but frequent injections are a serious draw-back in practice.

The observation obtained in animal experiments that certain compounds of steroid hormones and fatty acids are more slowly absorbed from the oil deposits than the free hormones has been used clinically with much success. The prolonged physiological effect of these esters was demonstrated for oestrogenic substances as far back as 1931 by *Butenandt*. The prolongation of the effect was originally believed to be due to a slow saponification of the esters in the blood stream, but the fact that the superiority of the esters to the free hormones is lost when they are given intravenously, proves that a delayed absorption rate must be the decisive factor. The question has been treated thoroughly and systematically in the classic investigations of *Deanesly & Parkes* (1937, 1938), *Miescher et al.* (1937), and *Pedersen-Bjergaard* (1939), where further references may be found. The effectiveness of the esters is dependent on the length of the acid side chain. The most satisfactory compound of testosterone has been found to be testosterone propionate, which combines a high biological activity with a fairly prolonged effect, and this compound is the most widely used of the testosterone esters.

It is, however, possible to approach the physiological conditions still more closely by administering the hormones as dry substances either by injections of a suspension of crystals or by implantation of compressed tablets. This therapeutically important achievement can be traced back to the excellent investigations of *Deanesly & Parkes* (1937) on the various factors influencing the effectiveness of administered hormones. The effectiveness of testosterone propionate was found to be enhanced by increasing the amount of oil, since the

absorption was slower from the larger oil deposits. When dealing with some other androgenic substances less soluble in oil it was found that the more concentrated oily solutions were more effective. The explanation of this phenomenon was that the substances crystallized out shortly after the injection and that the absorption from the crystals was a slow process. When implanting compressed tablets it was furthermore possible to determine the rate of absorption fairly accurately by removing, cleaning, drying and weighing the residue of the tablet at the end of the experiment.

The rate of absorption from the tablets is dependent on the chemical composition of the tablet, on its form (disc-shaped, cylindrical, or spherical) and on the degree of compression, as well as on the reaction of the tissues surrounding the tablet. *Deanesly & Parkes* (1937) found that tablets of testosterone propionate were absorbed more slowly than those of free testosterone, a finding which was confirmed by *Emmens* (1941) who, in experiments on rats, obtained a fair agreement between theoretically calculated and actually observed rates of absorption from disc-shaped tablets. The absorption from the tablets towards the end of their lives was somewhat higher than expected, presumably because erosion of the tablets resulted in relative increase of the surface.

Loeser (1940) examined the fate of subcutaneously implanted steroid hormone tablets in man and found the rate of absorption greatly dependent on the site of implantation, the absorption of some tablets being impeded by the development of a tough fibrous layer surrounding them.

It may perhaps be appropriate to emphasize that when the number of uniform tablets implanted under identical conditions is increased, the amount of substance released is increased, but the duration of absorption is not prolonged.

The duration and intensity of the effect of crystalline suspensions of desoxycorticosterone acetate have been determined by *Meier, Gasche & Frey* (1946) in experiments with adrenalectomized dogs and rats. From these interesting investigations it appears that the injection of crystalline suspensions

were superior to the injection of oily solutions both as regards the duration of effect and the utilization of the hormone. The significance of the shape of the crystals, their size and absolute amount was also clarified.

Beside the above-mentioned methods of administration of steroid hormones, the percutaneous and oral applications have been used clinically. The absorption of steroid hormones through intact skin is evident from the asymmetrical development of the mammary gland obtained by the one-sided inunction of alcoholic solutions or ointments containing oestrogenic substances. The quantitative aspect of the percutaneous absorption of testosterone propionate was the subject of some clinical investigations by *Foss* (1939); when administered to a castrated man, 2 to 3 times as much hormone was needed when given as an ointment as when injected intramuscularly in oily solutions. For alcoholic solutions the relation of the percutaneous dose to the parenteral dose was 6:1 if the same clinical effect was to be obtained. *Tager & Shelton* (1941) observed only a very slight effect after several months' treatment of a young hypogenital man with 14 mg. testosterone propionate daily in the form of ointment inunctions. When a local effect is desired, the percutaneous application of hormones may be useful, but in other cases the inconvenience of the inunctions and the poor utilization of the active substances limit the practical usefulness of this mode of administration.

Testosterone given orally is far less effective than given intramuscularly, the ratio of the oral to the intramuscular dose being claimed to be 10:1 to 30:1 (*Miescher & Tschopp*, 1938, *Emmens*, 1939, and *Emmens & Parkes*, 1939). In the above-mentioned castrate *Foss* (1939) found the ratio to be 20:1. The effectiveness of orally administered 17-methyltestosterone is comparatively high, and this compound is widely used clinically in the form of linguettes.

In animal experiments it is not difficult to obtain suitable test objects when investigating the effects resulting from changes in the method of hormone administration, e.g. the

cornification of the vaginal epithelium, the enlargement of the vaginal epithelium, the enlargement of the capon comb, the survival of adrenalectomized animals, etc., but in man we are confined to less exact criteria of response e.g. penile growth, pubic hair development and sexual potency. On the basis of the clinical demonstrable improvement in several men suffering from hypogenitalism, *Biskind, Escamilla & Lisser* (1941) concluded that the effect of subcutaneously implanted testosterone tablets lasts for an average of 7 weeks.

It is obviously desirable to possess an objective method for studying the absorption of the steroid hormones in man. As the administration of testosterone and other androgenic preparations is accompanied by an increased urinary excretion of androgenic substances and related compounds, this excretion might offer a means of estimating their rate of absorption. Disregarding a few investigations carried out before the methods of extracting the androgenic substances in the urine were efficient, it was not until 1939 that *Cook, Hamilton & Dorfman, Dorfman & Hamilton, and Callow, Callow & Emmens* succeeded in demonstrating an increased excretion of steroid substances in the urine of men treated with testosterone propionate. The substances were measured by biological methods («androgenic substances») and by Zimmermann's colorimetric reaction for »17-ketosteroids«. It has been possible to identify at least some of the compounds, and it may be regarded as beyond doubt that androsterone and its stereoisomeride aetiocholanolone are direct degradation products of testosterone.

Callow, Callow & Emmens (1939) demonstrated an increased urinary excretion of 17-ketosteroids, and of androgenic and oestrogenic substances as a result of injections of 50 mg. testosterone propionate or more per week in 6 men. *Hoskins et al.* (1939) treated four hypogenital men with 25 mg. testosterone propionate, injected intramuscularly daily for 9—14 days, and found an increase in the androgen excretion from 2—16 I.U./24 hours before the treatment to 44—115 I.U./24 hours during treatment; the high levels disappeared

shortly after the last injection. *McCullagh et al.* (1939) examined the androgen excretion in several normal and hypogonadal men after testosterone propionate injections. The effect of small doses (5—10 mg.) lasted for 24 hours and that of larger doses for several days. Subnormal values were obtained in normal men shortly after the increased excretion. This depression is thought to be due to a decreased endogenous androgen production as the result of the inhibitory effect of testosterone upon the hypophyseal gonadotrophin secretion. *Hamblen et al.* (1939) performed 17-ketosteroid determinations on the urine of women treated with testosterone propionate in single doses of 10—100 mg. and in series of up to 10 injections. From the rise in the excretion it was calculated that from 8 to 71 per cent of the testosterone propionate administered was excreted as 17-ketosteroids. In two castrated men *Dorfman & Hamilton* (1941) tested the androgen excretion on day-old chickens' comb after administration of testosterone propionate. After oral administration of 10 mg. tablets, increased androgen levels were already found in the course of the first 2 hours, but not after 48 hours. After intramuscular injections of oily solutions, the increased excretion could be traced for 7 days. Implantation of testosterone propionate tablets in the form of 6.5 mm. long rods weighing 15 mg. each resulted in high excretion values for 5 or 7—8 weeks when the total dose was 90 or 280 mg., respectively.

As to the metabolism of androgens in the organism and the chemical identification of their degradation products, reference is made to the publications of *Callow* (1939), *Callow & Callow* (1940), *Dorfman & Hamilton* (1940), *Schiller, Dorfman & Miller* (1945), *Dorfman, Horwitt, Shipley et al.* (1947), *Dorfman, Wise & Shipley* (1948), *Miller & Dorfman* (1948) and *Devis & Férin* (1948). We should like to mention here, however, that not all androgenic substances are excreted as 17-ketosteroids. *Frame, Fleischmann & Wilkins* (1944) found that testosterone, testosterone propionate, androsterone and dehydroandrosterone were excreted as 17-ketosteroids, whereas 17-methyltestosterone, 17-ethyltestosterone and several

other 17-methylcompounds were not excreted as such. *Reifenstein et al.* (1945) confirmed the inability of methyltestosterone to raise the 17-ketosteroid content of the urine, and found on the contrary, decreased values (hypophyseal inhibition).

OWN INVESTIGATIONS

In the Radium Centre, Copenhagen, the usual treatment of patients suffering from mammary carcinoma has since 1947 been supplemented by treatment with testosterone propionate (*Kaae*, 1949). The large quantities of hormone required made it highly desirable to carry out the treatment in as economical a manner as possible, that it to obtain the best utilization of the hormone. The question then arose, whether treatment with oily solutions, suspensions of crystals, or tablet implantations was to be preferred, and what effect the difference in the method of administration would have on the dose and frequency of administration. A mere evaluation of the clinical effect of the treatment was not likely to serve as a guide, and hence we decided to investigate what information could be obtained by estimating the 17-ketosteroid excretion in the urines of the patients under treatment.

Technique

The chemical determination of the urinary 17-ketosteroids was preferred to the biological method of assay because of its higher accuracy, the short time involved in the procedure, and the fact that an almost unlimited number of analyses could be carried out. The analyses were made in the *Hormone Department of the State Serum Institute*, a limited number of the first specimens of urines being examined according to the standard routine method used at that time (*Hamburger*, 1948 a, b), though the »micro-methods« (*Hamburger & Rasch*, 1948) were used in most cases, i. e. either the benzene-method or the ether-method. Since there is no essential difference between these methods, the method by which a particular analysis was performed is not recorded.

The ether-method is as follows:

1/50 of a 24-hour urine is hydrolyzed by boiling on an electric hot plate for 25 min. with 10 vol. per cent of 40 per cent H_2SO_4 . After cooling the urine is extracted once by vigorous shaking with ether in a separating funnel. The ether extract is extracted once with saturated NaHCO_3 solution, twice with 2 N NaOH solution and twice with water. After drying with dehydrated Na_2SO_4 , the ether extract is filtered and evaporated to dryness. The residue is taken up in 0.80 ml. of absolute alcohol. A colorimetric reaction is then performed in duplicate according to the method of *Callow, Callow & Emmens* (1938), 0.20 ml. of the alcoholic extract being mixed with 0.20 ml. 2 per cent alcoholic meta-dinitrobenzene and 0.20 ml. 2.5 N alcoholic KOH . The mixture is left in the dark for 60 min. at a temperature of 25°C , and diluted with 9.4 ml. of absolute alcohol. The colour is measured in a Coleman spectrophotometer at 530 $\text{m}\mu$ and 470 $\text{m}\mu$ and by means of a nomogram for correction for un-specific chromogenic urinary compounds, the 17-ketosteroid excretion is calculated as mg./24 hrs.

Preparations and injections

The following preparations have been used: testosterone propionate in oily solution, with 25 mg./ml. or 50 mg./ml. (= »Perandren« ampoules), testosterone propionate in crystalline suspension with 50 mg./2 ml. (= »Perandren« crystalline ampoules), testosterone propionate tablets, 100 mg. (= »Perandren« implantation tablets), cis-testosterone dissolved in oil + benzylalcohol, 20 mg./2 ml. , and a dry powder of crystalline (free) testosterone.*)

The oily solutions and the crystalline suspensions were injected intramuscularly in the region of the hip; they were given immediately after the last specimen of urine of a 24-hour collection had been voided, usually at 7 o'clock in the morning. The tablet implantations were made subcutaneously in the abdominal region. In the *Radium Centre* all injections and tablet implantations were given by one of the authors (S. K.).

*) All the preparations were generously supplied by the *Ciba Limited*, Copenhagen, to whom we should like to express our sincere thanks.

Patients and collection of urines

Most of the patients were women, hospitalized at the *Radium Centre* for treatment of mammary carcinoma. The general health condition of these in-patients was usually rather poor, while the less debilitated women were treated as out-patients. Accurate collection of 24-hour urines was found to be impossible in the case of the out-patients and was abandoned after some trials. Furthermore the investigation included some women, hospitalized for combined X-ray and radium treatment of uterine cervical carcinoma. These patients were in good condition but yet hospitalized sufficiently long for the collection of urine. Since, in these cases, the testosterone propionate was given only for experimental purposes, the doses of the hormone and the duration of treatment had to be rather moderate in order to avoid any virilizing effects. The investigation also includes several experiments conducted by one of the authors (C. H.) on himself.

The collection of 24-hour urines continuously for weeks or months was very difficult when dealing with the debilitated patients suffering from mammary carcinoma. Thus, inability to void the urine at a fixed hour, cases of severe diarrhea, residual urine, etc. made the collection unreliable. Out of 17 patients with mammary carcinoma, no less than 11 women were unable to collect their urine satisfactorily and the results had to be discarded. In the cases to be reported below, the collection of urine has probably been reliable although it is evident that in many of the patients the daily fluctuations in the 17-ketosteroid output are considerably greater than those found in the experiments of C. H. on himself. Several urines had to be discarded because of technical failure in the collection.

The experimental subjects can thus be divided into 3 groups:

1) Three patients with uterine cervical carcinoma, in whom the Perandren injections were given exclusively for experimental purposes; (case records No. 1—3).

2) Six patients with mammary carcinoma, operated as well

as inoperable cases, all having osseous metastases (case records No. 4—9).

3) One healthy male subject (C. H.), 43—45 years old from whom about 300 24-hour urine specimens were collected in the course of 2 years and assayed for 17-ketosteroids in order to determine the daily variations and the effect of various hormone administrations.

Case records

No. 1 (J. 49652). Woman 57 years old. Six partus, the last one 25 years ago. Menopause at 43 years. Diagnosis: Cancer colli uteri, stage III. Treatment: combined X-ray and radium therapy 2/10—10/11—48. General condition good. (Fig. 1).

No. 2 (J. 49825). Woman 55 years old. Eight partus, the last one 12 years ago. Menopause 6 months ago. Diagnosis: Cancer colli uteri, stage III. Treatment: combined X-ray and radium therapy 16/10—23/11—48. General condition good. (Fig. 2).

No. 3 (J. 49621). Woman 31 years old. Three partus, the last one 10 months ago. Regular menstruations. Diagnosis: Cancer colli uteri, stage I. Treatment: combined X-ray and radium therapy 7/10—13/11—48. General condition good. (Fig. 3).

No. 4 (J. 49772). Woman 72 years old. Four partus, the last one 32 years ago. Menopause at the age of 56 years. Right sided cancer mammae, untreated during the first 12 years. In Oct. 1948 there was a large, exophytic and ulcerative mammary cancer and wide-spread osseous metastases. M. D.: Carcinoma solidum. Perandren treatment 8/8—48—26/1—49. The general condition was poor, somewhat alternating; after a transient improvement, the condition was aggravated and the patient died on 28/2—49, 15 days after the last 17-KS analysis. Autopsy: wide-spread metastases to liver, right pleura, and the bones. (Fig. 8).

No. 5 (J. 48034). Woman 57 years old. Eight partus. Menopause at 45 years. Three and a half year before, radical mastectomy for carcinoma mammae M. D.: Carc. scirrhusum. Later X-ray treatment for metastases to the right axilla and left-sided supraclavicular glandular metastases. Oct.—48: extensive osseous and pulmonary metastases, and local recidive. Perandren treatment 8/10—48 to 26/1—49. From the beginning of the treatment her condition was rather poor and after a transient improvement, the patient got worse, had spontaneous fractures of left femur and tibia, and died on 28/2—49, one month after the last Perandren injection and 8 days after the last 17-KS analysis. (Fig. 9).

No. 6 (J. 30128). Woman 56 years old. Three partus. Menopause 51 years old. Five years previously: radical mastectomy for carcinoma mammae. M. D.: Carcinoma adenomatosum et scirrhosum mammae et gland. axill. In the course of 1947—1948 several series of X-ray treatment for metastases. June—48 metastases to columna, costae, cutis abdominis and left pleura. X-ray treatment 21/4—8/5—48 and Perandren treatment. The general condition was good. (Fig. 5).

No. 7 (J. 31062). Woman 56 years old. Four partus. Menopause at the age of 49 years. Five years previously: radical mastectomy for carcinoma mammae. M. D.: Carc. solidum mammae et axill. About 2 years ago X-ray treatment for metastases to columna and pelvis. Perandren injections (crystals) from 12/12—47 to 18/2—48 and implantation of 400 mg. Perandren tablets on 19/2—48. July—48 wide-spread osseous and some small cutaneous metastases. The general condition fair. The Perandren treatment was resumed and 7 tablets of 100 mg. were implanted subcutaneously in the abdominal region. No tablets or parts of tablets were expelled. (Fig. 10).

No. 8 (J. 49629). Woman 71 years old. Three partus, the last one 28 years ago. Menopause at the age of 54 years. The patient had a previously untreated double-sided mammary cancer with wide-spread osseous metastases. M. D.: Carcinoma scirrhosum. Perandren treatment, including implantation of 15 perandren tablets of 100 mg. subcutaneously in the abdominal region. No tablets or parts hereof were expelled. In the beginning of the treatment the general condition was rather good, later on it changed for the worse, there was a spontaneous fracture of the right femur, 18 days after the implantation of the tablets. Before the discontinuation of the observation period, the condition was considerably aggravated. (Fig. 11).

No. 9 (J. 45912). Woman 55 years old. One abortion 12 years ago, no partus. Menopause 47 years old. Fifteen years ago: radical mastectomy for carcinoma mammae. M. D.: Carcinoma solidum. X-ray treatment for a metastasis to the left supraclavicular region. Osseous metastases in Dec.—48. The general condition good. Perandren treatment from 13/12—48. (Fig. 12).

Results

The 17-ketosteroid excretion found during and after the administration of testosterone propionate (TP) and a survey of the treatments are shown in the diagrams (Figs. 1 to 12). They are all drawn to the same scale, the black columns indicating the 17-KS excretion in mg. per 24-hours. An empty space between the columns means that the urine was dis-

carded. The white wedges are symbols for injections of Perandren in oily solution, and the black wedges for injections of crystalline suspensions. Tablet implantations are marked with a black circle, and percutaneous application of testosterone with a black rectangle. The figures along the abscissae represent the days of the observation period. Referring to these diagrams it will suffice here to comment on the investigations and to draw certain general conclusions from the results.

1) *The depression of the hypophyseal function.*

It is a well-known fact that the hypophyseal gonadotrophin production is inhibited by injection of oestrogenic and androgenic substances. This inhibition involves the growth hormone and the thyrotrophic hormone. The effect upon other hypophyseal hormones, including the adrenocorticotrophic hormone is more uncertain. As, however, the 17-KS excretion is depressed as a result of testosterone treatment in both men and women, and as the adrenal cortex probably is the sole source of production of the 17-KS in the female organism, the treatment must have an inhibitory effect on the secretion of the adrenocorticotrophic hormone.

In the following paragraphs the decrease in the 17-KS excretion, caused by the testosterone inhibition of the hypophyseal function will be referred to as the »*hypophyseal inhibition*«, while the »*basal excretion*« means the endogenous 17-KS production. The »*extra excretion*« stands for the difference between the observed 17-KS values and the basal excretion.

The hypophyseal inhibition creates some difficulties in the calculation of the extra excretion, because it is not possible to know with certainty on which day of the treatment the diminution on the basal excretion begins, and how much it amounts to. The hypophyseal inhibition probably varies in different individuals and with the intensity of the treatment. One of the experiments of C. H. on himself shows the inhibition quite clearly (Fig. 6). After 5 daily injections of 25 mg. Perandren the 17-KS excretion was diminished on the 6th day

of treatment, the decrease amounting to about 5 mg./24 hrs. Immediately after discontinuation of the injection the daily excretion was also about 5 mg. lower than before the treatment; the pre-treatment level was reached in the course of one week. In some instances a single intramuscular injection of TP in oil is sufficient to produce the hypophyseal inhibition, e. g. 100 mg. in case No. 6 (Fig. 5) and 50 mg. in case No. 8 (Fig. 11).

In most cases we have calculated the extra excretion, partly with the assumption that no hypophyseal inhibition has taken place, and partly assuming that the greatest decrease of the 17-KS values observed after the treatment had been present during the whole period. The true value must then be found somewhere between these limits. As the molecular weight of TP is about 20 per cent higher than that of the 17-KS (e. g. androsterone, and aetiocholanolone), the calculations of the amount of TP degraded to 17-KS have involved a correction for the different molecular weights.

2) *Single intramuscular injections of testosterone propionate in oily solution.*

Case No. 1 (Fig. 1). The 17-KS excretion rose from an average basal excretion of 4.2 mg./24 hrs. to 46.8 mg. in the first 24 hours after the injection of 200 mg. Perandren. The increased excretion lasted for 5 days. The hypophyseal inhibition was moderate, and the extra excretion amounted to 85 mg. when the calculation involved the inhibition, otherwise it was 79 mg. According to these values 51 per cent, or 48 per cent of the TP was excreted as 17-KS.

Case No. 2 (Fig. 2). The injection of 200 mg. Perandren brought about a rise in the excretion from an average of 4.9 mg./24 hrs. to 33.9 mg. in the first 24-hour period. On the next three days the values were: 11.6, 13.5 and 6.4 mg. No hypophyseal inhibition was apparent in this case. Altogether 27 per cent of the TP was calculated to have been excreted as 17-KS, but the comparatively low value on the 2nd day suggests that some urine must have been lost.

Case No. 3 (Fig. 3). From a basal excretion of 6.3 mg./24 hrs. the 17-KS excretion increased to about 40 mg. in the first and second days and the high level was maintained for four days. The extra excretion amounted to 84 mg., corresponding to 50 per cent of the testosterone propionate injected.

Case No. 9 (Fig. 12). The injection of 200 mg. Perandren caused a rise in the excretion from 1.6 mg. to 52.0 mg. on the first day and the high level persisted for 3 or 4 days. The extra excretion equaled 40 per cent of the testosterone propionate injected.

Experiments by C. H. on himself (Fig. 4). The 17-KS excretion was determined for 13 days prior to the injection of 50 mg. Perandren and was on an average 12.6 mg./24 hrs. The extra excretion on the first two days was 30.3 mg., that is 72 per cent of the injected TP. Two weeks later the injection of 150 mg. Perandren was followed by a 4 days' period of increased 17-KS values and an extra excretion amounting to 63 mg., corresponding to a 50 per cent excretion. The first day's urine was collected as 6 samples at exactly 4 hourly intervals. An increased excretion was already found in the first 4-hour urine, and the maximal excretion in the next specimen. After both injections the high excretion was followed by values 2 to 3 mg. below the average pre-treatment value.

Case No. 6 (Fig. 5). An increased excretion occurred for 4 days after the injection of 100 mg. Perandren, and 56 per cent of the injected TP were excreted as 17-KS. (If the effect of the hypophyseal inhibition is disregarded, the percentage excretion is 46). On the 15th day of the observation period the patient received 25 mg. Perandren, after which injection the excretion was increased for 2 days. According to the extra excretion, not more than 22 per cent of the TP was excreted as 17-KS, but the calculations are of course less reliable when the excretion is in the neighbourhood of the normal values.

Case No. 7 (Fig. 10). The general state of the patient was rather poor and the average basal excretion was not more than 2.0 mg./24 hrs. The increase caused by 50 mg. Perandren was

moderate, but lasted for 6 days. About 36 per cent. of the TP was excreted as 17-KS.

Case No. 8 (Fig. 11). This patient was also in a poor condition and the basal excretion was low (averaging 2.1 mg.). About 34 per cent of the 50 mg. Perandren was excreted as 17-KS, when the hypophyseal inhibition was taken into account, otherwise it was 22 per cent.

The above-mentioned observations show that a single intramuscular injection of Perandren in oil causes a rapid rise in the 17-KS excretion, and that the first 24-hour urine contains the highest amounts. The high excretion lasts for 2 to 6 days and from 22 to 72 per cent of the injected TP is excreted in the urine as 17-KS, on an average 44 per cent. No definite relation between the size of the dose and the percentage excreted was noticed.

3) *Repeated injections of testosterone propionate in oily solution.*

Experiments on C. H. (Fig. 6). The average daily excretion for a period of 6 days before the injection was 13.7 mg. Daily injections of 25 mg. Perandren were given for 8 days. During the treatment the excretion rose gradually and reached about 27 mg. on the 3rd day, and this value was maintained for 3 days. In spite of continued injections the excretion then fell to about 22 mg., probably as a result of the hypophyseal inhibition. The accurate collection of the urine and the fact that the fluctuations in the daily excretion in this experimental subject are rather small, made worth while a detailed calculation of the mechanism of the absorption and excretion of the injected TP. If we suppose that the percentage excretion of each 25 mg. dose is 29, 19, 14 and 0 in the course of 4 days, and that the hypophyseal inhibition commences when 5 times 25 mg. are given and amounts to 4 or 5 mg. per 24 hours, we obtain a very close agreement between calculated and observed 17-KS values.

Case No. 4 (Fig. 8). The patient received 100 mg. Perandren every other day, altogether 17 injections in this series. The excretion was rather irregular, probably because of difficulties in the collection of the urine. It is, however, clear that the highest values occurred in the first 24 hours after each injection. The total extra excretion amounted to 7.6 mg./24 hrs. that is 18 per cent of the TP injected.

Case No. 5 (Fig. 9). The first treatment consisted of daily injections of 25 mg. Perandren for 11 days. The basal excretion was on an average 1.3 mg.; the values rose moderately and reached a maximum in the 3rd day but were afterwards rather irregular. The average extra excretion after the 3rd day was 4.3 mg./24 hrs., corresponding to 27 per cent of the injected amount of TP. Later on (from the 30th day) 100 mg. TP was given every other day, altogether in 6 injections. The excretion showed alternating high and lower values, the highest excretion being found in the first 24 hours after each injection. The extra excretion is difficult to calculate because the effect of a preceding injection of Perandren crystals had not yet disappeared. Our experience with the other injections of crystal suspensions, however, allows of a rough estimate which shows that 18 per cent of the 600 mg. TP was excreted as 17-KS.

According to these experiments it is possible to obtain a fairly uniform daily 17-KS excretion, and presumably also a fairly constant absorption, by giving daily injections of TP in oily solutions, whereas the excretion is alternately high and low when the injections are given every other day.

4) *Single intramuscular injections of crystal suspensions of testosterone propionate.*

Case No. 1 (Fig. 1). The 17-KS excretion increased gradually and reached a maximum (12.9 mg.) on the 5th day after one injection of 200 mg. Perandren crystals. The level then decreased gradually and the pre-treatment values were reached

on the 14th day. As, however, no signs of any hypophyseal inhibition were apparent in the last urine examined (16 days after the injection) it is reasonable to assume that some slight absorption from the crystals was still occurring, just enough to compensate for the depression of the endogenous secretion. The total extra excretion in the course of the 16 days amounted to 56 mg. (The missing value for the 3rd day being obtained by interpolation). This would indicate that 34 per cent of the TP was excreted as 17-KS. Presupposing a hypophyseal inhibition of the same magnitude as after 200 mg. in oily solution, the figure would be 45 per cent, that is almost the same percentage as after the injection of TP in oily solution.

Case No. 2 (Fig. 2). A rapid increase in the 17-KS excretion took place after one injection of 200 mg. Perandren crystals, and the maximum (20.7 mg.) was reached on the 3rd day (the urine from the 4th day was lost). The peak was followed by a gradual decrease in the values which reached the pre-treatment values 10 days after the injection. The extra excretion was of a magnitude which corresponded to 53 per cent of the injected TP, that is at least as much as after the above mentioned injection of oily solution (27 per cent), even if attempts are made at correction for the supposed loss of urine on the 2nd day.

Case No. 3 (Fig. 3). This patient received one injection of 500 mg. Perandren crystals, and the 17-KS excretion was already considerably increased in the first 24-hour urine reaching the maximal value (21.0 mg.) on the 3rd day. The excretion was gradually decreased and the pre-treatment values were reached on the 12th day. As no signs of hypophyseal inhibition were found, the absorption was probably not completely finished at this time. The total extra excretion amounted to 37 per cent of the injected Perandren, if the calculations are based upon a hypophyseal depression of the same order as after the 200 mg. oily injection. It is, however, likely that the inhibition after 500 mg. Perandren was even more marked and that some absorption occurred after the observation period. The percentage of TP excreted as 17-KS is thus thought to

be in the neighbourhood of that observed after an injection of 200 mg. in oily solution, i. e. 50 per cent.

A comparison of the changes in the 17-KS excretion caused by a single injection of TP in oily solution and as a crystal suspension shows that in the former case the highest excretion occurs within the first 24 hour, and that the main part of the TP is eliminated in the course of the first 2 or 3 days, while the maximal excretion after crystal injections is found at the 3rd or 5th day and increased excretion persists for 9 to 14 days, or probably even longer. The percentage of hormone eliminated as 17-KS is the same with both these methods of administration.

5) *Repeated injections of crystal suspensions of testosterone propionate.*

Case No. 4 (Fig. 8). Two days after repeated treatment with Perandren in oily solution the patient received a series of crystal injections, at first 200 mg. every other day. The excretion decreased after the first injection because the slow absorption of the crystalline material could not counter-balance the rapid elimination of the hormone dissolved in oil. Not until 4 injections had been given did the excretion reach a plateau. After the 6th injection the interval between the injections was increased, the injections being given every fourth day. From the 66th day of the observation period and onwards 350 mg. were given once a week. The average excretion in the interval between the injections was fairly constant, 17 to 22 per cent of the substance being excreted as 17-KS (during the oily injections the percentage was 18). It is a very striking fact that toward the end of the observation period the 17-KS excretion fell gradually in spite of the continued treatment, the amount of TP eliminated as 17-KS being 17, 9 and 5 per cent, respectively, in the last 3 weeks. This inability of the organism to metabolize TP to 17-KS may be the result of the patient's worsening condition. Another explanation might be the development of very large liver

metastases which appeared at the same time, the damaged liver being unable to take part in the metabolism of the TP.

Case No. 5 (Fig. 9). During the first 41 days of the investigation, the patient excreted from 18 to 27 per cent of the TP in oil as 17-KS. From the 42nd day and during the rest of the 138 days of observation, she was treated exclusively with injections of crystalline Perandren. At first a series of 200 mg. injection was given every other day; in the latter half of this series, that is when the excretion had reached a plateau, the extra excretion corresponded to 26 per cent of the TP. Then 200 mg. was given every fourth day and later 350 mg. once a week. The percentage of TP excreted as 17-KS was from 18 to 23, yet with a somewhat declining tendency (after the last injection only 13 per cent.) Like the above-mentioned patient her condition became worse and large liver metastases developed.

Case No. 6 (Fig. 5). From the 28th day of the observation period after the previous treatment with 2 Perandren injections in oil and one crystal injection, the patient received 100 mg. crystalline Perandren every other day, altogether 9 injections. The 17-KS increased gradually, reached a maximum (27.4 mg.) on the first day after the last injection, and decreased thereafter. By the 14th day after the last injection the excretion was lower than before the commencement of the injections. The patient left the hospital, but the urine was analyzed 7 and 16 days later; both specimens had a subnormal 17-KS content. Of the 900 mg. injected 41 per cent was eliminated as 17-KS.

These observations demonstrate that it is possible to get a fairly uniform 17-KS excretion (reflecting a uniform absorption of the hormone) by weekly injections of crystal suspensions of TP. The daily variation in the 17-KS seemed to be quite independent of the days of injection and are probably due to inaccuracies in the collection of the 24-hour urines. The average daily excretion of 17-KS is the same when TP is given as repeated injections of oily solutions or of crystal

suspensions (e. g., 100 mg. in oil every other day and 200 mg. crystals every fourth day, or, 50 mg. in oil daily and 350 mg. crystals once a week).

6) *Implantation of testosterone propionate tablets.*

Case No. 7 (Fig. 10). After subcutaneous implantation of 7 Perandren tablets of 100 mg. each, the 17-KS excretion increased from the 2nd day and reached in the course of a week a plateau which was kept up for at least 23 days when the continued analyses were interrupted. A specimen of urine was examined 28 days after the implantation and the 17-KS content was at the pre-treatment level. On the 42nd day the excretion was subnormal (hypophyseal inhibition). The possibility cannot be ignored that absorption from the tablets occurred beyond the 28 days, the daily amount absorbed being, however, so insignificant that it merely compensated for the decrease in the endogeneous 17-KS production. The daily excretion was fairly uniform during the first 23 days, and within this period the extra excretion was 36 mg. When estimating the hypophyseal inhibition necessary to cause a daily decrease of 1.5 mg., the extra excretion is calculated as 48 mg. It is, however, as mentioned above, possible that the absorption continued until the 42nd day; thus the excretion would equal 14 per cent of the implanted tablets. The same patient excreted 36 per cent of the oil dissolved Perandren, and the utilization of the tablets must therefore have been rather incomplete in this case.

Case No. 8 (Fig. 11). After the effects of a single injection of 50 mg. Perandren in oil had disappeared, a total amount of 1500 mg. Perandren tablets were implanted subcutaneously. The operation was followed by increased 17-KS values and the moderately increased level was maintained for about 5 weeks. Then a gradual decrease commenced and on the 46th day the content was as low as 1.0 mg. The total extra excretion corresponded to 14 per cent of the implanted tablets. Of the first injection of 50 mg. in oil, 34 per cent was eliminated in the form of 17-KS, but after subsequent injections of 200

and 100 mg. Perandren in oil, the percentage excretion was only 8. The ability of the organism to metabolize TP to 17-KS thus seemed to be impaired gradually as the state of the patient grew worse. Ascites developed but liver metastases were not demonstrated, and the patient died at home without autopsy. It is most likely that the absorption from the tablets did not occur after the 46th day and that about 50 per cent of the substance was absorbed within this period of time.

A fairly constant absorption from the subcutaneously implanted tablets of TP was thus obtained for 3 to 4 or 5 weeks in these cases. But the absorption seems to be incomplete, and hence it is necessary to implant about twice as much hormone as when using oily solutions or crystal suspensions.

7) *Percutaneous administration of testosterone propionate and of testosterone.*

The investigation of the percutaneous absorption was the purpose of two experiments of C. H. on himself. Of an alcoholic solution of *testosterone propionate* (20 mg./ml.) about 2 ml. were rubbed into the skin of the abdomen and the inside of the thighs in the morning and in the evening for 4 days, the total dose being about 320 mg. The 17-KS excretion did not exceed the maximum of the subject's normal values. The absorption of TP through the skin must therefore have been very low. Two months later an experiment was performed using *free testosterone* in alcoholic solution (50 mg./ml.). In the course of 5 days 20 ml. were rubbed into the skin, that is a total dose of 1000 mg. As evident from Fig. 7 the 17-KS excretion increased from an average value of 11.5 mg. to about 20 mg., and this level was maintained during the treatment. After discontinuation of the application the 17-KS gradually decreased to and below the normal values. The gradual decrease is thought to be due to a continued absorption from the deep parts of the hair follicles. The total extra excretion was calculated to amount to 54—66 mg., corresponding to 5—7 per cent of the testosterone applied. When

injecting oil solutions of TP it had been shown that the experimental subject eliminated between 50 and 72 per cent as 17-KS, i. e. 10 times as much as by the percutaneous administration of testosterone.

8) *Oral administration of testosterone propionate.*

In an experiment by C. H. on himself the effect of oral administration of 100 mg. testosterone propionate on the 17-KS excretion was examined. The content of two Perandren crystal ampoules was swallowed with water on an empty stomach. Already 2 hours after the ingestion of the hormone, an increase of 17-KS was found in the first specimen of urine, the excretion being 1.43 mg. per hour. (In control investigations the corresponding figures determined on two other days were found to be 0.48 and 0.53 mg. per hour for the same two hours of the morning.) The maximal excretion per hour occurred in the next urine specimen, covering the period from 2 to 5 hours after the administration of the hormone. In the first 24 hours' urine the total amount was 22.0 mg. 17-KS, that is 8.9 mg. above the average excretion before the experiment. In the next 24-hour urine the extra excretion was only 1.6 mg. Thus, 12 per cent of the orally administered TP was eliminated as 17-KS. As about 60 per cent of the parenterally administered TP was previously found to be excreted as 17-KS, only one fifth of the hormone was utilized when given orally.

9) *Intramuscular injection of cis-testosterone.*

The stereoisomeride of testosterone, Δ^4 -androstene-3-on-17(β)-ol = cis-testosterone, is remarkable for the fact that it is almost devoid of androgenic effects. It possesses, however, a marked antagonistic effect upon the ability of oestradiol to produce hypophyseal tumours (*Selye*, 1947). With the possibility in view of using this compound in the treatment of mammary carcinoma we examined its excretion in the urine.

Case No. 9 (Fig. 12). The patient had eliminated 40 per cent of testosterone propionate, administered intramuscularly in oily solution. When the effect of this injection had disappeared, 200 mg. of cis-testosterone, dissolved in oil with benzylalcohol added, were given intramuscularly. In the first 24-hour urine, but only in this, was there an increased 17-KS content, the extra excretion amounting to merely 5.3 μ g. or 2—3 per cent of the cis-testosterone.

DISCUSSION

It has been demonstrated in the above investigations that testosterone propionate and testosterone are metabolized in the organism and excreted in the urine as 17-ketosteroids. The percentage of the hormone excreted in this form varies with the individual and is dependent on the general health of the subject, the excretion being lower in debilitated patients. The liver seems to play a role in the process. A remarkably low 17-KS excretion was thus found after treatment with testosterone propionate in two of the patients with carcinomatous metastases in the liver. In four male subjects suffering from cirrhosis of the liver *Lloyd & Williams* (1948) found no increase in the amount of 17-ketosteroids in the urine after injection of 50 mg. testosterone propionate, whereas two had significant increase in the total excretion of androgens.

On an average, somewhat less than half the amount of TP is excreted as 17-KS, the lower and upper limits in our investigations being 5 and 72 per cent, when administered as intramuscular injections of oily solutions or of crystal suspensions. These values are in complete agreement with the findings of *Hamblen et al.* (1939), who found from 8 to 71 per cent. *Pelzer et al.* (1948) recovered on an average 40 per cent of the TP as 17-KS.

The absorption, metabolism and excretion occurs very rapidly. After oral administration of 100 mg. TP an increased 17-KS excretion was demonstrable in the course of the first two hours. *Dorfman & Hamilton* (1941), using the biological

method of assay, found a similar rapid rise in the excretion of androgenic substances. After a single *intramuscular* injection of TP in oily solution, the highest excretion occurred in the first 24-hour urine, the amount decreasing more or less abruptly during the next 2 or 5 days. It was possible to obtain a fairly uniform 17-KS excretion by daily injections, and the rate of absorption must be assumed to be fairly constant too. When the injections are given every other day the excretion is alternately high and low, presumably reflecting an uneven rate of absorption.

After intramuscular injections of TP in *crystal suspension* it is usually possible to demonstrate an increased 17-KS excretion in the first 24-hour urine. The highest values are found on the 3rd to the 5th day and the increased excretion lasts for about 2 weeks, when the pre-treatment level is reached. It was possible to obtain the same smooth 17-KS excretion (and rate of absorption) by weekly injections of the crystals. These figures naturally depend on the size of the crystals, but in our observations we have found no indication that the size of the crystal varies very much in the different Perandren batches.

Subcutaneously implanted testosterone propionate tablets are absorbed more slowly than the crystals and the increased excretion can be traced for 4 to 7 weeks. In this respect too, our results are in agreement with those of *Dorfman & Hamilton* (1941) who found an increased androgen excretion (biological assay) for 5 to 8 weeks, and with those of *Biskind et al.* (1941) who obtained a clinically demonstrable effect for an average of 7 weeks after the implantation. The utilization of the tablets seems to be less efficient than that of the crystals or oily solutions presumably because the reaction of the tissues surrounding the tablets may sometimes interfere with the absorption (cp. *Loeser*, 1940). If the absorption of TP from the oil deposits is complete, not more than about 50 per cent of the tablets were absorbed in the two cases reported here, in which 7 and 15 tablets, each of 100 mg. TP were implanted subcutaneously.

The *percutaneous absorption* of TP and of free testosterone is very incomplete. The application of 320 mg. TP to the skin of a normal male in the course of 4 days was not accompanied by any definite rise in the 17-KS excretion, and calculations showed that less than 8 per cent of the substance applied was absorbed. Free testosterone in alcoholic solution seems to pass through the intact skin somewhat better than the propionate. There was a marked increase in the 17-KS excretion when 1000 mg. testosterone was rubbed into the skin of the same experimental subject in the course of 5 days. It was calculated that about 10 per cent of the testosterone applied was absorbed. Foss (1939) stated that in a castrated man the percutaneous dose of TP was 6 times as large as the intramuscular dose. This small difference may be due to individual variations, to difference in technique, or to the fact that the evaluation of the clinical effects is not a very objective criterion of response.

Orally administered TP was in our experiments absorbed at a higher rate than when given percutaneously to the same person, as the 17-KS excretion corresponded to a 20 per cent absorption of the hormone. Foss (l. c.) had to give 20 times as much TP orally as parenterally in order to obtain equal clinical effects. The reason for this discrepancy might be found in the fact that the hormone absorbed from the intestinal canal must pass through the liver by means of the hepatic portal system before reaching the general blood circulation. The TP escaping the hepatic inactivation might be degraded to biologically less active compounds such as androsterone and dehydroandrosterone or to biologically inert substances such as aetiocholanolone. In that case the clinically effective dose of hormone must necessarily be higher than expected from the rate of absorption, as calculated on the basis of the 17-KS excretion.

The stereoisomeride of testosterone, *cis-testosterone*, was eliminated as 17-KS only to a very slight extent (2—3 per cent). In this respect it behaves according to the evidence in the literature like *methyltestosterone*. We are not able to

offer any explanation for these differences between testosterone and testosterone propionate on the one hand, and cis-testosterone and methyltestosterone on the other.

An inhibitory effect upon the hypophyseal production of gonadotrophic and adrenocorticotrophic hormones, as evident from subnormal 17-KS values after the cessation of the testosterone administration, was observed in several cases even after not very large single injections of TP, or short series of treatments with ordinary therapeutic doses. This observation is of interest when compared with the findings of *McCullagh & Hruby* (1949) that very large amounts of testosterone were required in order to inhibit the gonadotrophic overproduction in eunuchoid men.

SUMMARY

The purpose of the investigations was primarily to decide whether determinations of the 17-ketosteroid excretion in the urine might serve as an objective means of finding out the most economical mode of administration of testosterone propionate.

The experimental subjects were 6 women suffering from cancer mammae, 3 women with cancer colli uteri and one healthy man in his middle forties. The investigations reported consist of the 17-KS-analyses of 524 twenty-four-hour specimens of urines, and the analyses were performed consecutively for at least 18 and at the most 138 days.

The preparations used were Ciba's Perandren ampoules of 25 mg./ml. or 50 mg./ml., Perandren crystal ampoules of 50 mg./2 ml., Perandren implantation tablets of 100 mg., testosterone and cis-testosterone.

The methods of administration consisted of intramuscular injections of oily solutions or crystal suspensions, subcutaneous implantations of tablets, percutaneous applications of alcoholic solutions, and oral administration.

The technique of the 17-ketosteroid-analyses was the Calow modification of the Zimmermann reaction with correc-

tion for unspecific urinary chromogens. Most urines were assayed by a »micro-method« (cold ether extraction of 1/50 of the acid-hydrolyzed urine).

Results:

1) An increased 17-ketosteroid excretion was found for 1—2 days after oral administration of testosterone propionate; for 3 days after percutaneous application of testosterone in alcoholic solution; for 3—5 days after intramuscular injections of testosterone propionate in oily solution; for an average of 12 days after injections of testosterone propionate crystal suspensions and for 4 and 7 weeks after subcutaneous implantation of 7 and 15 testosterone propionate tablets of 100 mg.

2) If the ultimate absorption of testosterone propionate from intramuscular oil deposits, which presumably is complete, is rated as 100, the absorption of the crystals was also 100, that of the implanted tablets 50, of orally administered crystals 20, of percutaneously applied testosterone 10, and of testosterone propionate less than 8.

3) A fairly uniform daily absorption could be obtained when the intramuscular injections of oily solutions were given daily, the injections of crystal suspensions once a week, and the implantations of tablets once a month.

4) When the treatment is of long duration, the injections of crystal suspensions are preferable because of the comparatively prolonged absorption and the complete utilization of the hormone. The disadvantage of the oily injections is the necessity of daily injections, while the less complete utilization of the implanted tablets and the operation required must be regarded as draw-backs of the implantation technique. The percutaneous and the oral administrations are so uneconomical that they cannot be recommended. (The investigations do not include methyltestosterone, as this compound is not excreted as 17-KS.)

5) Cis-testosterone, administered intramuscularly, was ex-

creted as 17-ketosteroids only to a very slight extent (2—3 per cent).

6) An inhibition of the hypophyseal corticotrophin and gonadotrophin secretions, as reflected in subnormal 17-KS values after cessation of testosterone administration, was noticed in several instances.

7) The 17-ketosteroid analyses must be regarded as an important help in arranging therapy with testosterone propionate for individual subjects.

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Figs. 1—12.

Urinary 17-ketosteroids before, during and after treatment with testosterone preparations. Ordinates: 17-KS in mg. per 24 hours. Abscissae: days of observation period. A missing column indicates that the particular urine was lost. The white wedge-shaped triangles indicate an intramuscular injection of an oily solution, the black ones an intramuscular injection of a crystal suspension. A black circle indicates the implantation of tablets, and a black rectangle indicates the percutaneous application of an alcoholic solution. The figures above the symbols are the amount of substance in milligrams.

From the Endocrinologic Department of Serafimerlasarettet,
Stockholm, Sweden (R. Luft, M. D.)

THE EFFECT OF DESOXYCORTICOSTERONE
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Postural hypotension is a term used in the present work to describe a condition reported by *Bradbury & Eggleston* in 1925. Arterial orthostatic anemia denotes a clinical condition that has been described chiefly by the Swedish investigators *Bjure & Laurell* (1927), and corresponds to what *Schellong* (1936) called »hypotonic regulatory disturbance«. The latter is a type of postural reaction, frequently observed, and common in asthenic persons with poor tone of muscles and tissues.

¹⁾ The DCA used in the present study, viz. Percorten Ciba, was kindly supplied by Ciba Produkter A. B., Stockholm.

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In healthy subjects, in arterial orthostatic anemia, and in postural hypotension, the variations in blood pressure and pulse rate during the change from recumbent to erect position show the following characteristics:

In *healthy subjects* there is often a slight rise in the pulse rate and diastolic blood pressure, with unchanged systolic pressure (*Asmussen, Christensen & Nielsen, 1939*).

In *orthostatic anemia*, the systolic pressure shows a moderate fall, with or without a simultaneous rise in the diastolic pressure. The result is a lowered pulse pressure. At the same time, the pulse rate is markedly accelerated (*Bjure & Laurell, 1927*, and *Knudsen, 1943*). These changes are due to increased tone of the sympathetic nervous system which counteracts the hydrostatic force due to the changes of position. Actually, fluent transition exists between the reactions of a healthy and an orthostatic person when changing from recumbency to a standing position (*Asmussen, Christensen & Nielsen, 1939*). In both cases, the cardiac output decreases (*Lindhard, 1913*; *Hickam, 1948*). In orthostatic subjects the reduced cardiac output is further accentuated by such factors as low peripheral tone (*Bjure & Laurell, 1927*; *Mayerson & Bursch, 1940*), large varicose veins (*Jeffers, Montgomery & Barton, 1941*), and pregnancy (*Hansen, 1942*).

In a previous investigation (*Luft & Sjögren, 1948*), we were able to demonstrate the ability of DCA and sodium chloride to prevent the orthostatic reaction with a simultaneous increase of the blood volume.

In *postural hypotension*, the sympathetic compensation of the hydrostatic changes is absent. This is due to an organic disease of the brain stem or spinal cord (*Stead & Ebert, 1941*; *Ellis & Haynes, 1936*). In these cases, the systolic and diastolic blood pressures are subjected to a momentary fall during the change from a recumbent to an erect posture, the pulse rate being maintained at the same level.

The hypertensive effect of DCA and sodium chloride in postural hypotension has previously been emphasized (*Luft & Sjögren, 1948*). The postural response remained unchanged,

though the patients were relieved of their symptoms because the fall in blood pressure started from a higher level in the erect position.

Gregory in 1945 demonstrated a case of »orthostatic anemia« successfully treated with DCA. However, a differential diagnosis cannot be made between this disease and postural hypotension on the basis of the description of this case.

On the other hand, the effect of DCA on the blood pressure in other conditions has been studied only to a limited extent. For instance, in adrenal insufficiency, the low blood pressure may be brought up to normal and sometimes even to a hypertensive level (*Ferrebee, Ragan, Atchley & Loeb, 1939*).

In healthy subjects with a normal blood pressure, the administration of DCA and sodium chloride (10 mg. and 5—10 gm. daily, respectively) does not as a rule cause a change in the blood pressure, though a slight increase was sometimes registered after a few weeks (*Perera, Knowlton, Lowell & Loeb, 1944*). In uncomplicated essential hypertension, DCA may cause a rapid rise of blood pressure. This effect has, so far, only been described in essential hypertension (*Perera & Blood, 1947; Perera, 1948*).

Clinton & Thorn (1943) found that in healthy subjects DCA increased the plasma volume by an average of 17.5 per cent. Sodium chloride alone, increased the plasma volume by 6 per cent.

Is the hypertension produced by DCA due to an increase of the blood volume? This is denied by *Perera & Blood (1947)*. They gave 10 mg. of DCA and 10 gm. of NaCl daily to ten subjects with normal blood pressure and 14 patients with essential hypertension. The blood pressure of the normal subject remained normal, while it was increased in the hypertensive subject. Both groups, however, showed the same changes of blood volume. DCA was administered only for one week in each group.

The question as to why DCA, potentiated by sodium chlo-

ride, causes an immediate rise of the blood pressure in essential hypertension still remains unsolved.

The present authors studied the changes of certain components that might be related to those of the blood pressure after long continued administration of DCA and sodium chloride. These examinations were made on three healthy subjects, two cases of postural hypotension and two of orthostatic anemia.

METHODS

Blood pressure measurements were taken every morning before breakfast. A Baum Mercury Manometer was used. The hematocrit readings and red blood cell counts were made by routine procedures. Determinations of blood volume were made with T. 1824, according to *Gibson & Evans* (1937). Inulin and diodrast clearances were determined according to *Smith et al.* (1938).

Before DCA was given, the patients were put on a hospital diet poor in salt (less than 3 gm. daily), the exact composition not being further analysed. After about ten days, DCA and sodium chloride were added. The experiments were terminated by a short period in which the sodium chloride and DCA supply was discontinued.

CASE REPORTS AND RESULTS

Case 1. F. A. No. 1244/48, 26-year-old man suffering from a ruptured intervertebral disc. For two weeks he received 20 mg. DCA and 10 gm. NaCl daily. There was a very slight rise in the blood pressure. The changes in body weight, water balance, hematocrit readings and red blood cell counts are shown in fig. 1.

Case 2. H. G. R. No. 949/48, 25-year-old man with traumatic epilepsy. Received 20 mg. DCA and 10 gm. NaCl daily for two weeks. There was only a very slight rise of systolic and diastolic blood pressure. See fig. 2.

Case 3. K. G. E. No. 582/48, 37-year-old man suffering from low back-ache. Administration of 20 mg. DCA and 10 gm. NaCl daily for 13 days resulting in only a very slight and non-significant change of blood pressure. For results, see fig. 3.

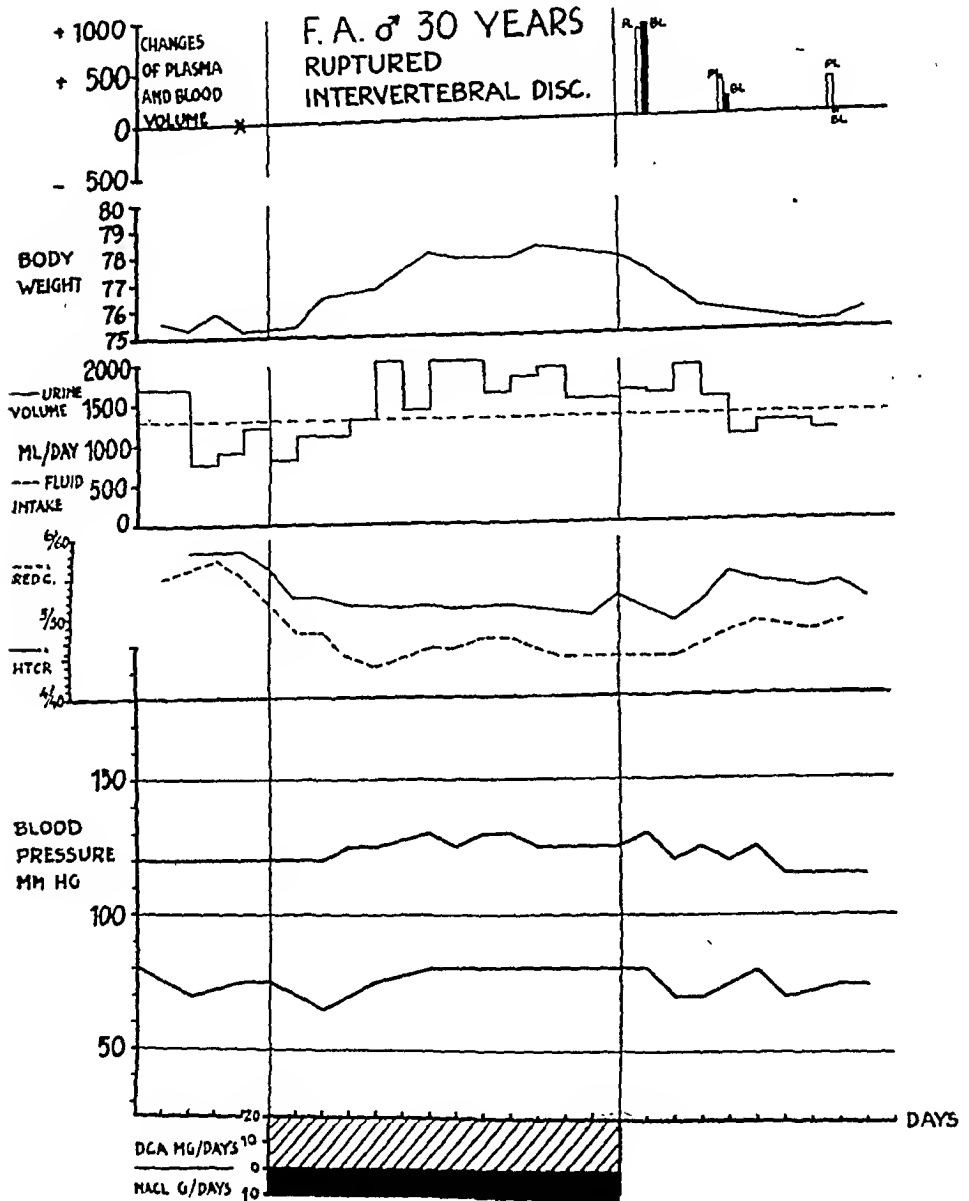


Fig. 1. Case 1. Male aged 30 years with ruptured intervertebral disc. Effect of DCA and sodium chloride on systolic and diastolic blood pressure, red blood count and hematocrit reading, fluid balance, body weight (in kg.), plasma and blood volume. The initial plasma and blood volumes are indicated with a cross on the line through zero; the changes (in ml.) from the initial values are indicated by an open (for plasma, PL) or filled (for whole blood, BL) column.

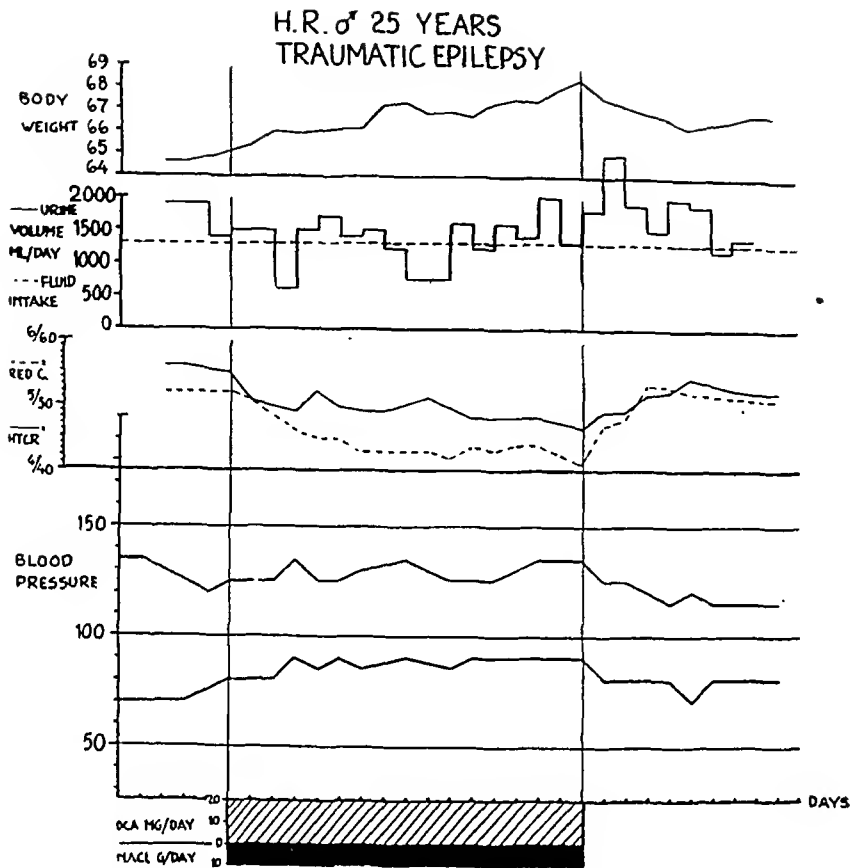


Fig. 2. Case 2. Male aged 25 years with traumatic epilepsy.
For legend, see Fig. 1.

Case 4¹). K. O. V. E. No. 234/47. Unmarried man, 47 years old. Symptoms of postural hypotension for over 10 years. Almost complete invalidism during the last year, could not remain standing up without fainting. Except for the abnormal blood pressure reaction (fig. 4), no pathological changes could be found. Neurological examination gave normal findings. Normal reaction on applying pressure to the carotid sinus. No clinical signs of adrenal cortical insufficiency (Cutler's and Kepler's tests negative). ECG normal on standing up, i. e. no orthostatic phenomena. WaR negative.

¹) For detailed case histories in Nos. 4—7, see Nordisk Medicin 40, 1764, 1948.

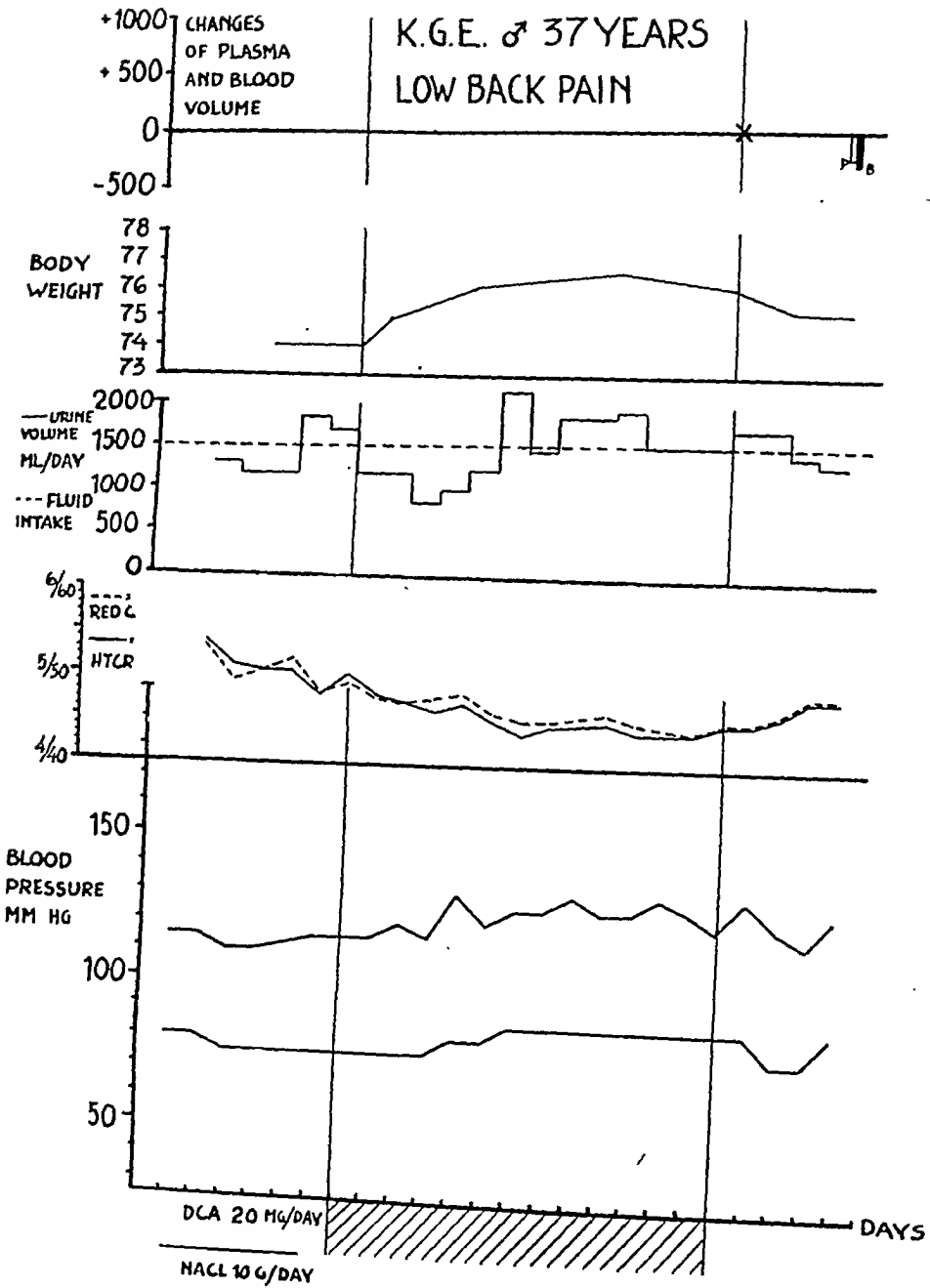


Fig. 3. Case 3. Male aged 37 years with low back-ache.
For legend, see Fig. 1.

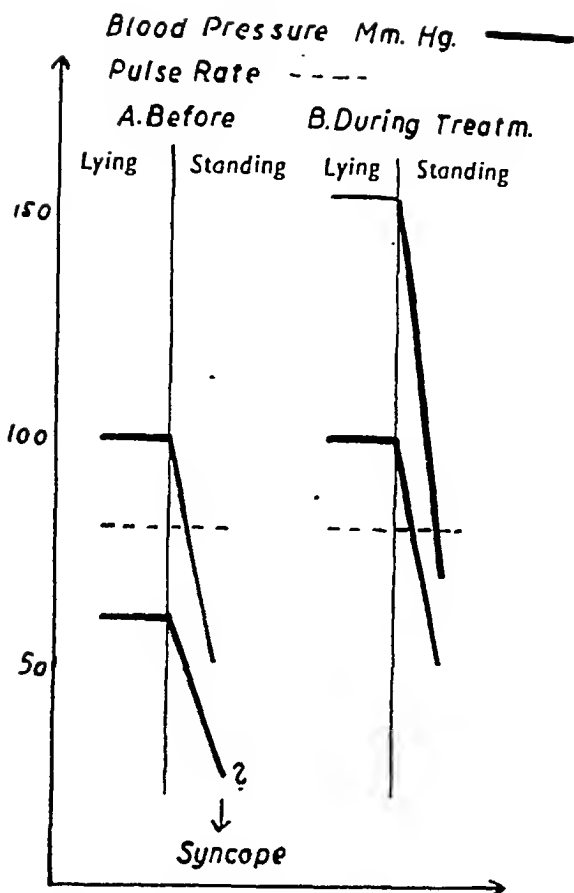


Fig. 4. Case 4. Male aged 47 years with postural hypotension. Blood pressure and pulse rate in supine and erect posture before and during treatment.

For two weeks the patient received 10 gm. NaCl daily with some slight change of blood pressure, from 110/70 to 120/80 mm Hg. During a second period, lasting 82 days, we administered 10 gm. NaCl and 20 mg. DCA daily. There was now a continued rise of blood pressure up to 165/100 mm. Hg. During this period the pressure remained at an average level of 155–160/100 mm. Hg. After the treatment, the initial pressure was regained within ten days.

The blood volume was determined during the last part of the experiment. The plasma volume showed a slight decrease towards the end of the DCA-period (fig. 5). After discontinuing DCA, we did not find any further decrease. In a previous investigation on this patient (Luft, Santesson & Sjögren, 1948), the plasma and blood volumes showed a significant increase when DCA was given.

DCA administration was followed by fluid retention (fig. 5). A new equilibrium was reached after about two weeks, while cessation of treatment on the whole did not involve any increase in the diuresis. The changes of body weight ran parallel to those of the water balance.

Inulin clearance gave lower values after medication had been withdrawn, being a sign of lowered filtration pressure. Diodrast clearance remained unchanged throughout the experiment, the value being fairly low, viz. 390.

The therapeutic effect is illustrated in Fig. 4. The postural type of reaction was unchanged. Nevertheless, the patient was relieved of the symptoms, as the fall in the blood pressure started from a higher level in the erect posture and did not reach the extremely

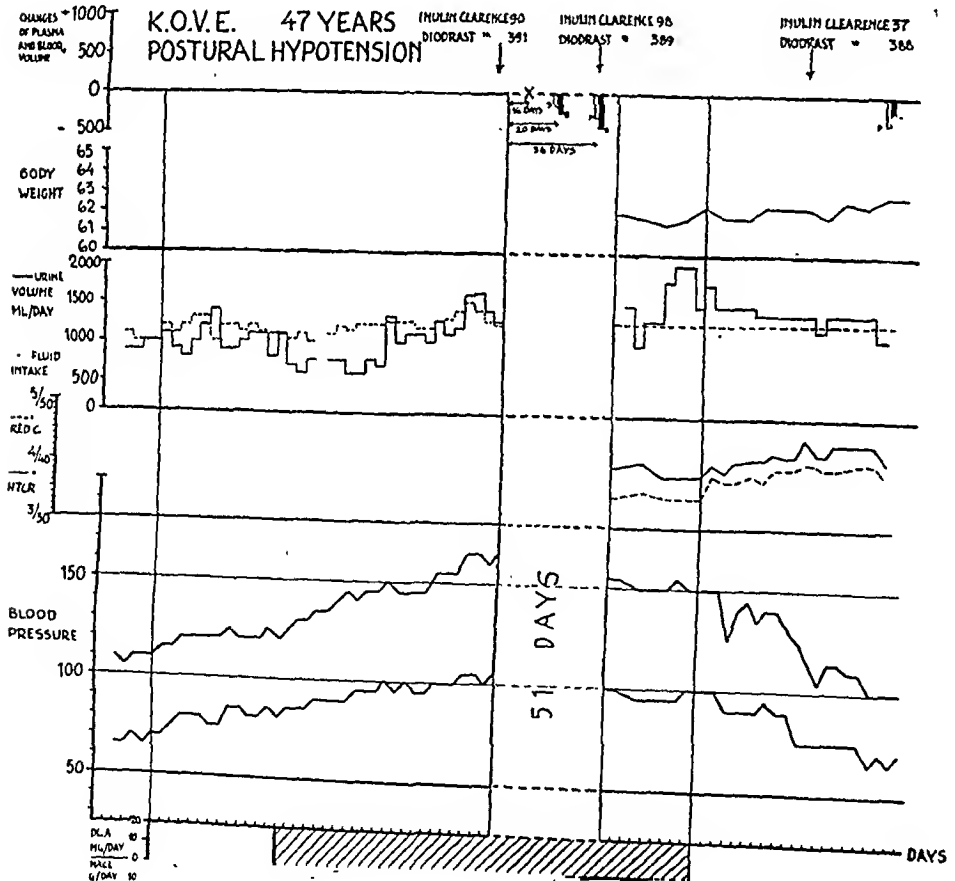


Fig. 5. Same patient as in Fig. 4.
For legend, see Fig. 1.

low values noted before treatment. The patient was able to resume his former work of a carpenter. On discharge, five 100 mg. tablets of Percorten were implanted. The implantation was repeated five months later.

Case 5. I. S. M. No. 403/48. Married woman, 53 years old. During the past two years she had fainting spells when standing, the spells being more frequent in the last months. On admission, she could only walk »doubled up«. The abnormal pressure reaction is seen in Fig. 6. Neurologic examination normal. WaR negative. ECG normal, no »orthostatic« changes. Normal reaction to carotid pressure. No clinical signs of adrenal insufficiency. Inulin clearance 77, diodrast clearance 444.

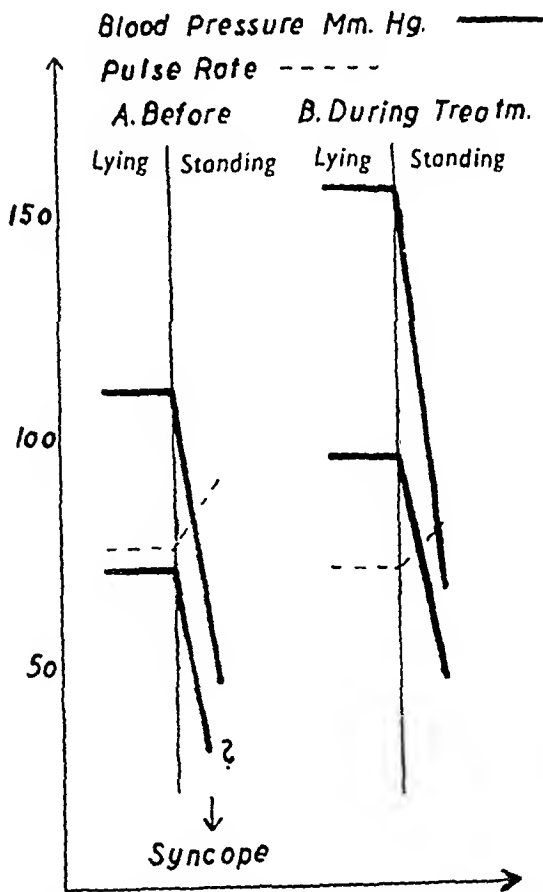


Fig. 6. Case 5. Female aged 53 years with postural hypotension.
For legend, see Fig. 1.

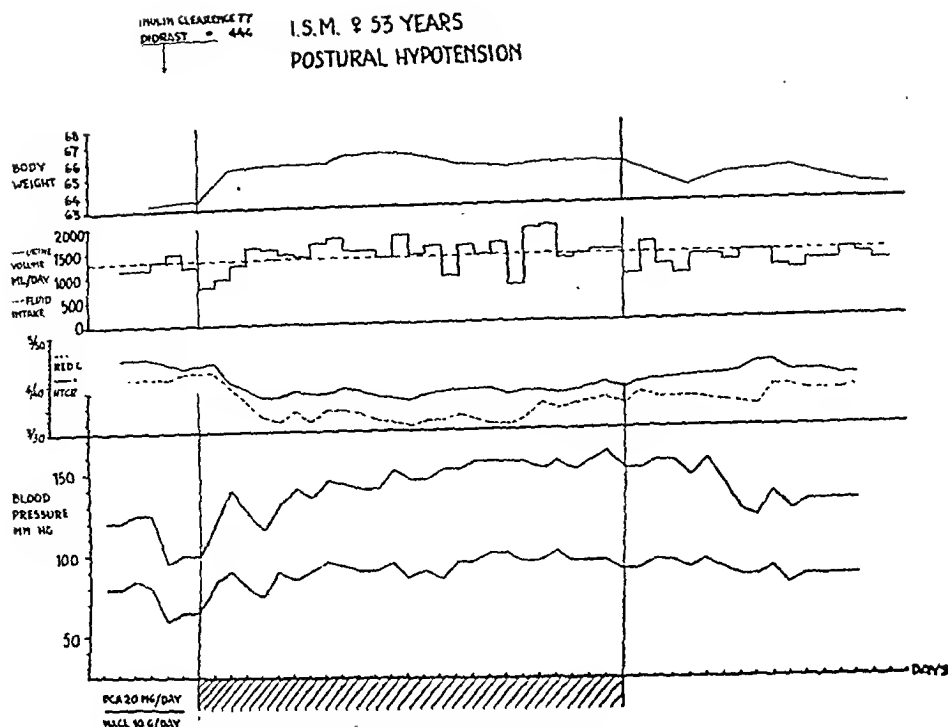


Fig. 7. Case 5.

Blood pressure and pulse rate in recumbent and erect posture, before and during treatment.

She received 10 gm. NaCl and 20 mg. Percorten daily for 27 days (fig. 7). A gradual rise of the blood pressure appeared. After 12 days, the blood pressure reached 155/95 mm. and remained at this level during the DCA administration. When treatment was discontinued, the blood pressure rapidly returned to its initial level.

No measurements were made of the blood volume. The changes in hematocrit readings and red blood cell counts were of the same type and magnitude as in Cases 1—3. No increase was registered in the diuresis after treatment had stopped. The body weight increased about 3 kg. during treatment.

The objectively measurable improvements are shown in fig. 6. As in case 4, the postural type of reaction remained, but the blood pressure did not fall to such extreme values in the erect position. There was a marked subjective improvement, and the patient could walk about freely, although she still felt a slight sensation when changing from a recumbent to an erect posture. On discharge, five tablets of Percorten, of 100 mg. each, were implanted.

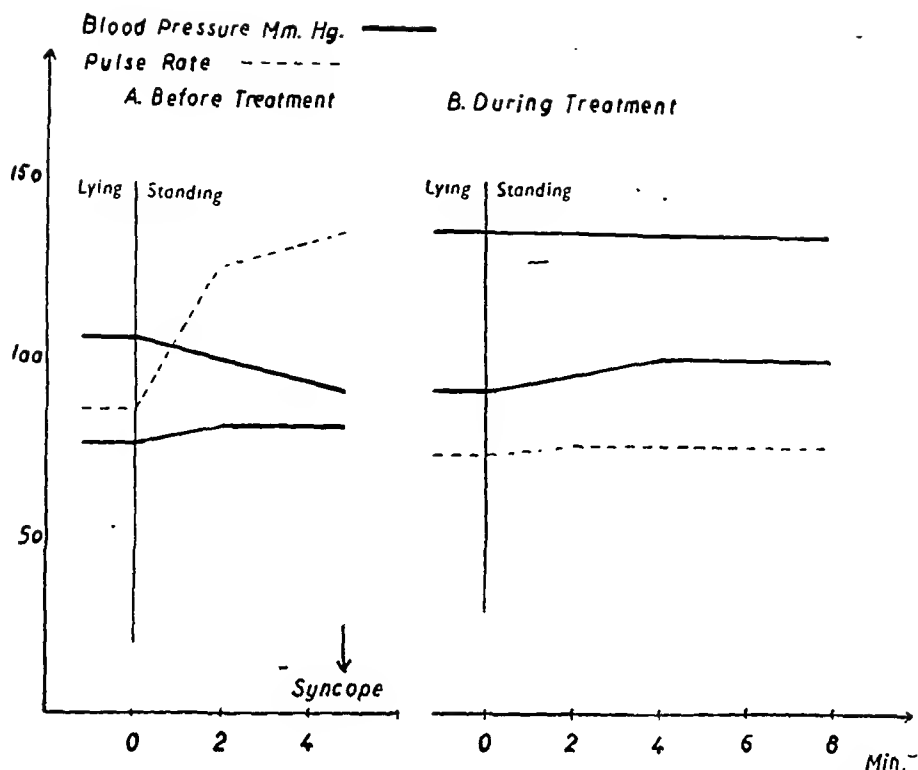


Fig. 8. Case 6. Female aged 31 years with arterial orthostatic anemia. Blood pressure and pulse rate in recumbent and erect posture, before and during treatment.

Case 6. E. L. B. No. 442/48. Married woman, 31 years old. For 10 years, severe headaches and dizziness when standing up. Increasing troubles after delivery in 1941 and 1945. Subsequently there were more frequent fainting spells. It was difficult for her to remain standing up, especially in the morning. She was a pale, slender woman. Neurological examination was normal. These were no clinical signs or laboratory findings indicating organic disorder.

ECG at rest normal. When standing up, increase of pulse rate from 85 to 135 within eight minutes. Simultaneously depressed ST-segments in leads II—II—IV, T₁ low, T₂ isoelectric, P-waves high in all leads.

Blood pressure and pulse rate in recumbent and standing positions, and orthostatic type of reaction are seen in Fig. 8.

The patient received 20 mg. Percorten and 10 gm. NaCl daily for 36 days (fig. 9). After two days of treatment there was a rise in blood pressure up to 120—130/85 mm. Hg from an initial value of 105/75.

The pressure remained at this level for the rest of the DCA period of treatment and rapidly returned to 105/75 mm. Hg. after the treatment.

The administration of DCA was rapidly followed by an increase of 26 per cent in the plasma volume and of 8 per cent in the total blood volume. The volume showed a slight fall towards the end of treatment. After discontinuing treatment, the volume returned to the initial level. DCA treatment also caused water retention, a new fluid equilibrium being reached within a week. At the same time, the body weight increased 3 kg., and the hematocrit readings and red blood cell counts were reduced. The NaCl administration caused a moderate increase of diuresis but no rise of the hematocrit reading. When the DCA administration ceased, there was a slight rise of the hematocrit readings and red blood cell counts, but no increase in the diuresis. The body weight was reduced by one kg. The inulin clearance was lowered with the fall in blood pressure after the end of the treatment, while the diodrast clearance remained unchanged.

The therapeutic effect is shown in Fig. 8 and was observed within one week of treatment. The orthostatic type of reaction disappeared,

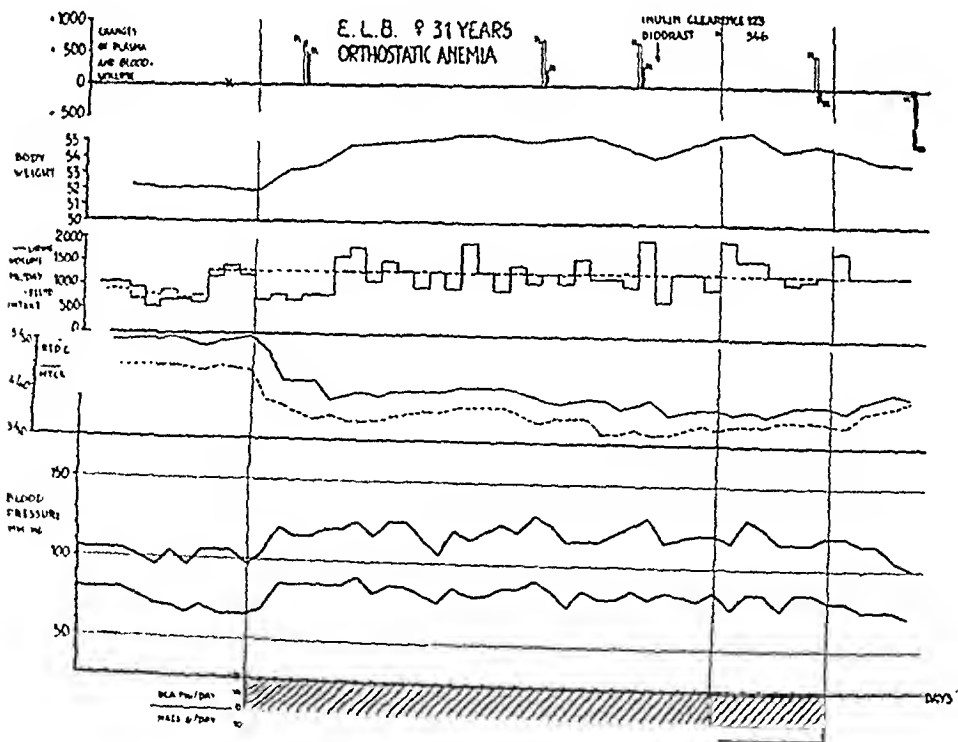


Fig. 9. Case 6.
For legend, see Fig. 1.

the orthostatic ECG changes were markedly reduced. The symptoms completely disappeared. Since discharge from the hospital the patient has been treated with Percorten in crystal suspension, 50 mg. intramuscularly every second week.

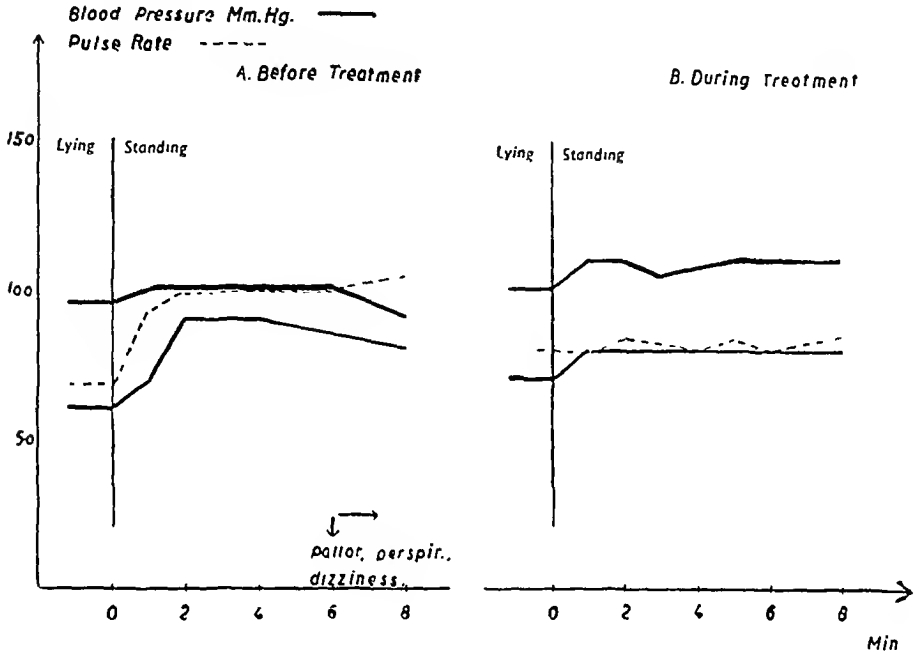


Fig. 10. Case 7. Female aged 34 years with arterial orthostatic anemia.

Blood pressure and pulse rate in recumbent and erect posture, before and during treatment.

Case 7. I. G. M. W. No. 312/48. Married woman, 34 years old. For the past four years, handicapped by orthostatic troubles, such as dizziness and tiredness on standing up, sometimes accompanied by fainting spells. It was difficult for her to remain standing. She was a slender, pale, asthenic woman. Neurological examination normal. No clinical signs of organic disorder. ECG at rest normal, on standing up, orthostatic changes of the same type as in Case 6. Blood pressure and pulse rate on lying down and standing up are shown in fig. 10.

Twenty mg. DCA and 5 gm. NaCl were given daily for 9 days (fig. 11). By this treatment the blood pressure increased, from 90/70 to 110/80 mm. Hg. The hematocrit readings and red cell counts were reduced. No closer examination was made. During a second period,

the patient received 20 mg. DCA and 5 gm. NaCl daily for 10 days with the same result as in the first period. The therapeutic effect is shown in Fig. 10. The patient had recovered completely and was discharged and instructed to take five sublingual tablets of 1 mg. each of Percorten daily.

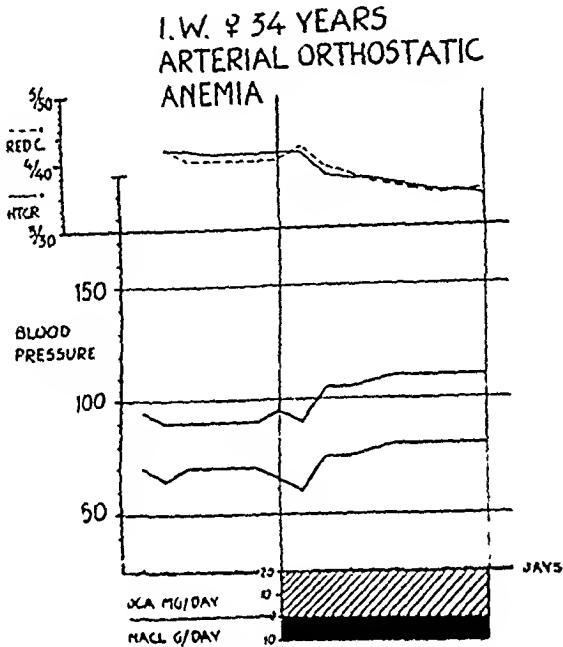


Fig. 11. Case 7.
For legend, see Fig. 1.

DISCUSSION

DCA and sodium chloride have been found to have a marked therapeutic effect in cases of postural hypotension and orthostatic anemia. In the former cases the effect was accompanied by a rise in blood pressure, i. e., 35—55 mm. Hg systolic and 20—40 mm. Hg diastolic, reaching a value of 155—165/95—105 mm. Hg. In the latter cases, the rise in the systolic pressure attained 25 mm. Hg, that of the diastolic pressure up to 15 mm. Hg, but the blood pressure did not in any instance reach higher values than 130/90 mm. Hg. Three »healthy« control subjects showed a blood pressure reaction of the same order of magnitude as did the group of orthostatic ane-

mia. The therapeutic effect in the cases of postural hypotension was evidently related to the rise in blood pressure, since the postural type of reaction remained unchanged. On the other hand, in arterial orthostatic anemia the therapeutic effect seemed due to a neutralization of the orthostatic type of reaction.

Previously, a hypertensive effect of DCA has only been demonstrated in a few cases of essential hypertension (*Perera, Knowlton, Lowell & Loeb*, 1944, *Perera & Blood*, 1947, and *Perera*, 1948).

The connection between adrenal cortical function and blood pressure regulation has formed the subject of extensive animal experiments during the past years. *Goldblatt's* investigations (1946) showed the significance of the adrenal cortex in experimental renal hypertension. The depression of the blood pressure produced in *Goldblatt's* animals by adrenalectomy, could be eliminated by DCA. *Selye*, and co-workers (*Selye, Hall & Rowley*, 1943), produced nephrosclerosis and hypertension in their animals by massive doses of DCA. *Zweifach et al.* (*Zweifach, Shorr, Baez & Rosenfeld*, 1947) demonstrated the role of the adrenal cortex and DCA in the production of a vasomotor principle in the kidneys.

The changes which occurred in the blood volume after DCA and NaCl administration were studied. In all the cases, a rapid fall was noted in the hematocrit readings and red blood cell counts, indicative of an increased plasma volume. This was confirmed by direct measurements of plasma and total blood volumes with T. 1824. The moderate increase of body weight (2—3 kg.) was a sign of water retention, as was also the discrepancy between fluid intake and urine volumes seen in some cases. These changes were of the same magnitude in the cases with raised blood pressure as in the others.

After therapy was discontinued the blood pressure rapidly fell to the original level in the cases of postural hypotension. However, the hematocrit readings and red blood cell counts showed only slight alterations, and the increase of diuresis

was but small in postural hypotension as well as in the other groups.

The results obtained offer strong evidence against the assumption that changes in blood volume should be chiefly responsible for the hypertensive effect of DCA in cases of postural hypotension.

Selye, Hall & Rowley (1943) observed changes in the renal parenchyma in his animals after the administration of NaCl and massive doses of DCA for a short period. In two of our own cases, 20 mg. DCA and 10 gm. NaCl were given daily for 36 (Case 6), 53 and 82 days (Case 4), respectively. An examination of the kidney function with inulin and diodrast clearance did not reveal any decrease in glomerular filtration but a slight decrease of renal plasma flow in these cases.

In a discussion of the mechanism of the hypertensive effect of DCA, the fact that glomerular filtration and renal blood flow were moderately lowered in both cases of postural hypotension must be taken into consideration. The possibility that the impaired renal blood flow may have some influence on the rise in blood pressure cannot be excluded.

A third explanation of the hypertensive effect of DCA may be found in a direct action on the vascular tone and, thereby, on the peripheral resistance. Such a mechanism might account for the therapeutic effect in orthostatic anemia. However, the question then arises, why was only a normal blood pressure and not hypertension obtained in these cases. Another explanation for the therapeutic effect in orthostatic anemia may, perhaps, be found in the increased blood volume after DCA administration.

SUMMARY

1. Desoxycorticosterone acetate (DCA) and sodium chloride had a marked therapeutic effect in two cases of postural hypotension and two cases of arterial orthostatic anemia. In the former, the postural type of reaction in the erect posture persisted, but the systolic and diastolic blood pressure did not

reach extremely low values during treatment. In orthostatic anemia, the changes in blood pressure and pulse rate disappeared during treatment.

2. The effect of DCA and sodium chloride, 20 mg. and 10 gm. daily, respectively, on the blood pressure, plasma and blood volume, body weight, water balance, hematocrit reading and red blood cell count was studied in the above cases, and in three young men with no signs of circulatory disturbances. In the cases examined, we found an increased blood volume on the same magnitude as previously described by *Clinton & Thorn* (1943) for healthy subjects.

3. In postural hypotension the treatment produced a significant rise in systolic as well as diastolic blood pressure.

4. There was no close correlation between the changes in blood volume and blood pressure in the cases examined.

5. The authors discuss the possible relation between the rise in blood pressure and renal function during DCA administration.

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ANNOUNCEMENTS

from the Endocrinological Societies

SWEDISH SOCIETY FOR ENDOCRINOLOGY

Meeting, Febr. 21, 1949.

- B. Berlin*: Paroxysmal hypertension. Report of a case.
Hj. Holmgren: Tissue culture and endocrinology (film) .
J. Möllerström: Metabolism of kalium in diabetes.
S. Björkman: Addison's disease. Report of a specially interesting case in respect of the etiology.
Hj. Wijnblad & *B. Engfeldt*: Hypoplycemia. Report of a case operated upon.

Meeting, April 25, 1949.

- R. Luft*: Gynaecomastia, adenoma of the adrenal cortex, hyperplasia of the adrenals and changes of the pituitary gland. Report of a case.
B. Sjögren: Anorexia nervosa and Morbus Simmonds.
R. Luft & *B. Sjögren*: Modern diagnosis and therapy in Addison's disease.
R. Luft & *B. Sjögren*: Renal function in endocrine diseases.
B. Swedberg: Hormones and experimentally produced tuberculosis.
A. Ljung: Electrocardiogram in hypo- and hypercalcaemia.

DANISH SOCIETY FOR ENDOCRINOLOGY

13. Meeting, May 2, 1949, Zoölogical Museum, Copenhagen.

- Chr. Hamburger* & *S. Kaae*: Influence of the mode of administration of testosterone propionate upon the 17-ketosteroid excretion in human beings.

14. Meeting, May 31, 1949, Zoölogical Museum, Copenhagen.

- G. J. van Oordt* (guest): Recent endocrinological investigations carried out in the Zoölogical laboratory at Utrecht.

Clinique médicale, Université de Louvain
(Prof. J. P. Hoet).

ASSOCIATION DU POLYVINYL-PYRROLIDONE À LA PITUITRINE, DANS LE TRAITEMENT DU DIABÈTE INSIPIDE

PAR

J. LEDERER

Le traitement du diabète insipide par la pituitrine n'est pas exempt de difficultés. D'une part les injections sous-cutanées ne provoquent leur action anti-diurétique que durant quelques heures (4 à 8 heures) d'où la nécessité dans les cas graves de les répéter (2 à 5 injections par jour) et elles éveillent souvent des malaises fort désagréables (pâleur, angoisse, céphalées, palpitations, diarrhées), d'autre part, l'absorption par la muqueuse nasale sous forme de poudre à priser peut ne pas être tolérée.

Claisse, Choay & Choay (1947 a, b) pour obvier à ces inconvénients ont eu l'idée d'associer les extraits post-hypophysaires à une substance retard, le polyvinyl-pyrrolidone ou sublosan.

Voici les résultats observés par l'application de cette méthode dans trois cas de diabète insipide où ont été comparées successivement la diurèse par 24 heures lorsque les malades recevaient uniquement de la pituitrine puis la pituitrine associée au polyvinyl-pyrrolidone.

Chez ces malades, la durée d'action de la pituitrine seule et celle de différentes doses de pituitrine associée au polyvinyl-pyrrolidone ont été également comparées.

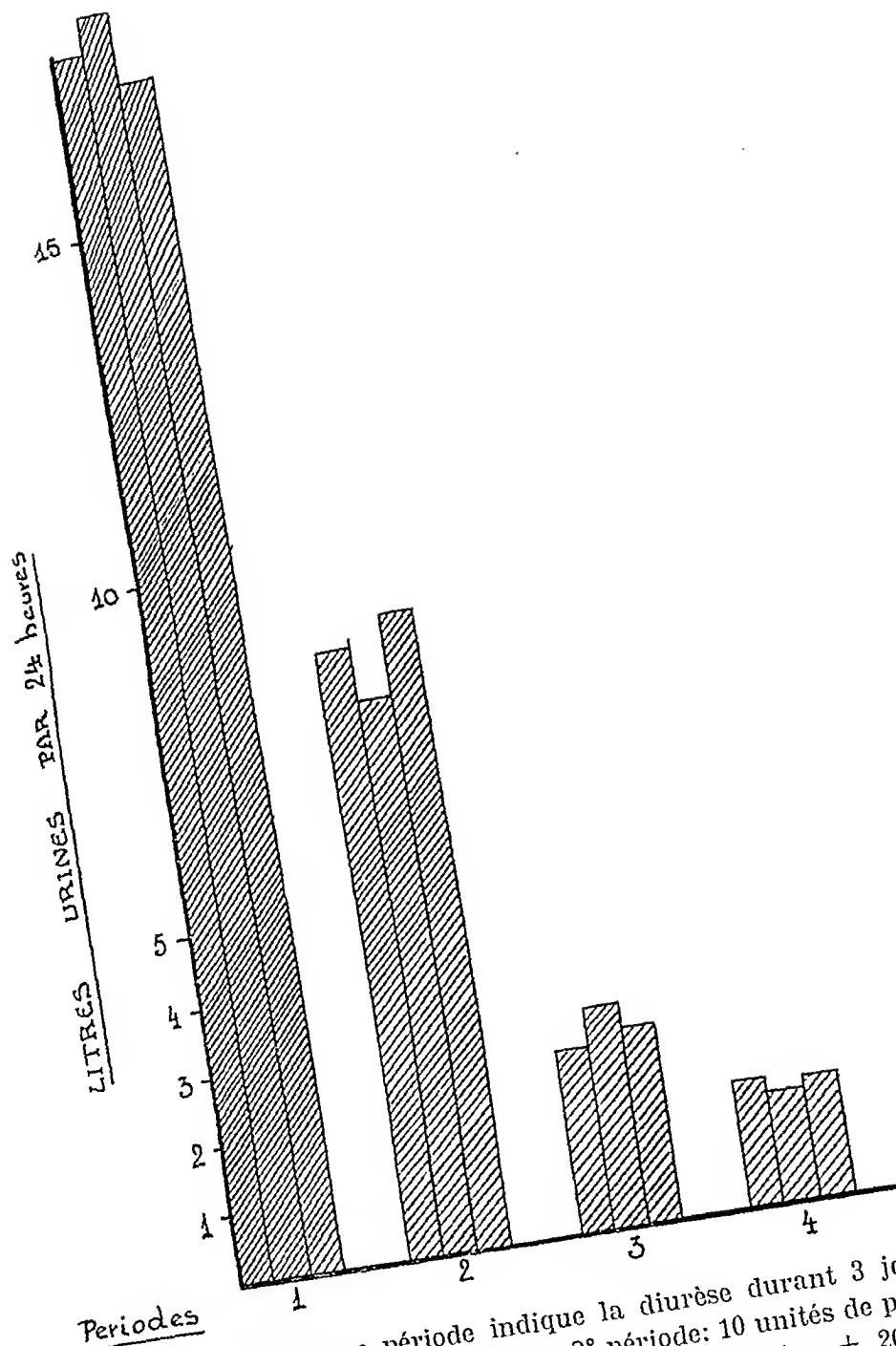
OBSERVATIONS

Cas 1. — Mme Jos. — 62 ans. — est atteinte de diabète insipide depuis 1 an environ. Il s'agit d'un diabète insipide grave dont la diurèse se situe aux environs de 17 l. par 24 heures. Ni l'examen elinique, ni les examens radiologiques, ni les recherches de laboratoire n'ont pu assigner une eause précise à l'écllosion de l'affection.

La malade est soumise aux injections d'extrait post-hypophysaire. Elle recoit 10 unités de pituitrine en injection sous-cutanée. Ces injections n'ont une durée d'action que de 5 à 6 heures et chaque injection déelenche des malaises importants: pâleur, palpitations, céphalées, diarrhées. Pour amener la diurèse entre 2 et 3 l., il faut faire à la malade, 4 injections quotidiennes de 10 unités de pituitrine. Devant l'ampleur des malaises ressentis à l'oecasion de chaque injection la malade refuse de faire plus d'une injection par jour de 10 unités de pituitrine, mais dans ces conditions, la soif et la polyurie ne sont calmées que quelques heures chaque jour et la diurèse par 24 heures se situe entre 8 et 9 l.

La malade s'adresse alors à l'extrait post-hypophysaire pour prise nasale. Celui-ci ne combat qu'imparfaitement et de manière inconstante la polyurie et rapidement la malade développe une allergie vis-à-vis de ce mode d'administration; chaque prise est suivie d'une violente crise d'asthme, aussi la malade renonce-t-elle rapidement à cette méthode pour reprendre l'injection sous-eutanée de 10 unités de pituitrine par jour. Les malaises provoqués par cette injection et son action insuffisante entraînant pendant la plus grande partie de la journée une soif dévorante, rendent la vie de la malade des plus inconfortables.

A ce moment la malade vient en observation dans le service où après étude de son eas, il est décidé de lui administrer chaque jour une injection sous-eutanée d'un mélange de 1 ml. de pituitrine contenant 10 unités Voegllin et 1 ml. d'une solution de polyvinyl-pyrrolidone à 20 p. 100. L'effet de ces injections est remarquable parce que d'une part elles n'éveillent plus aucun malaise et que d'autre part leur action sur la

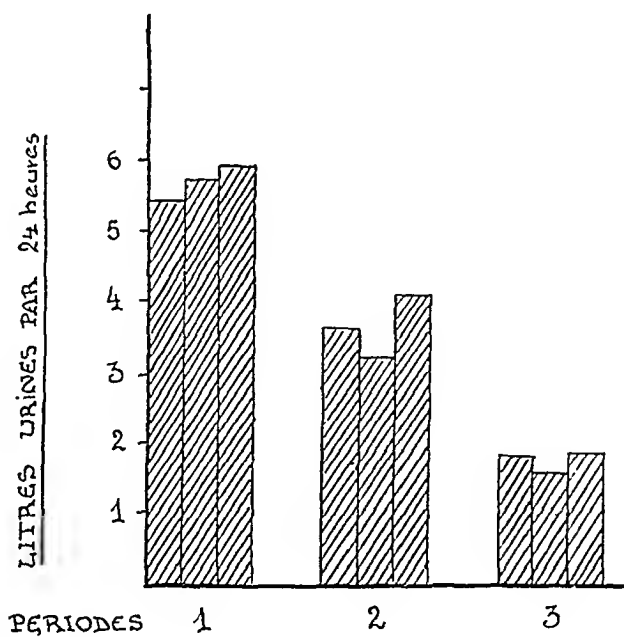


Graphique I. — Chaque période indique la diurèse durant 3 jours consécutifs. 1° période: sans traitement, 2° période: 10 unités de pituitrine tous les matins, 3° période: 10 unités de pituitrine + 20 cg. de polyvinylpyrrolidone (2 ml.) tous les matins, 4° période: 10 unités de pituitrine + 25 cg. de polyvinyl-pyrrolidone (1 ml.) tous les matins.

diurèse est beaucoup plus intense et prolongé. En effet par cette association, la diurèse par 24 heures tombe aux environs de 3 l., ce qui entraîne une énorme amélioration des conditions de vie.

La malade reçoit ensuite chaque jour une injection d'un ml. d'un mélange contenant par cc. 10 unités Voegtlin de pituitrine et 25 cg. de polyvinyl-pyrrolidone. Par cette association, la diurèse est réduite à moins de 2 litres par 24 heures, ce qui supprime toute sensation de soif anormale en ne provoquant aucun malaise.

Dans la suite est déterminée chez cette malade la durée d'action de différentes doses de l'association contenant par ml. 10 un. Voegtlin de pituitrine + 25 cg. de polyvinyl-pyrrolidone. Avec 1 ml. de ce mélange, la sensation de soif est calmée dans trois expériences successives durant 28 heures, 31 heures et 29 heures. Avec 2 ml. de ce mélange, la sensation



Graphique II. — Chaque période indique la diurèse durant trois jours consécutifs. 1° période: sans traitement, 2° période 10 unités de pituitrine tous les matins, 3° période: 10 unités de pituitrine + 20 cg. de polyvinyl-pyrrolidone (2 ml.) tous les matins.

de soif et l'exagération de la diurèse sont calmées dans deux expériences successives durant 41 heures et 43 heures. Avec 3 ml. de ce mélange, la sensation de soif et l'exagération de la diurèse sont calmées dans 3 expériences successives durant 52 heures, 54 heures et 49 heures.

Depuis 8 mois, la malade se fait tous les deux jours une injection sous-cutanée de 3 ml. du mélange contenant par ml. 10 unités Voegtlin de pituitrine et 25 cg. de polyvinyl-pyrrolidone. Ces injections ne lui donnent aucun malaise d'ordre général et provoquent une gêne douloureuse fort supportable durant 2 ou 3 heures au lieu d'injection. La diurèse se maintient grâce à ce traitement en dessous de 2 litres par jour.

Cas 2. — M. Van J. — 35 ans, est atteint de diabète insipide depuis 6 mois. La diurèse par 24 heures se situe entre 5,5 et 6 l. Aucun examen ne permet d'assigner une cause déterminée à l'éclosion de la maladie.

Le malade reçoit chaque matin 1 injection sous-cutanée de 10 unités de pituitrine. La soif et la polyurie sont diminuées durant 8 à 10 heures. mais chaque injection provoque chez le malade de l'angoisse et des palpitations et parfois une diarrhée. Sous l'effet de ce traitement la diurèse est d'environ 3,5 l. par jour.

Dans la suite, le malade reçoit chaque matin une injection d'un mélange de 1 ml. de pituitrine contenant 10 unités Voegtlin et de 1 ml. d'une solution de polyvinyl-pyrrolidone à 20 p. 100. Sous l'effet de cette association, la diurèse tombe à moins de 2 litres par 24 heures, faisant disparaître toute sensation anormale de soif. Le malade n'éprouve aucun malaise à la suite de ces injections.

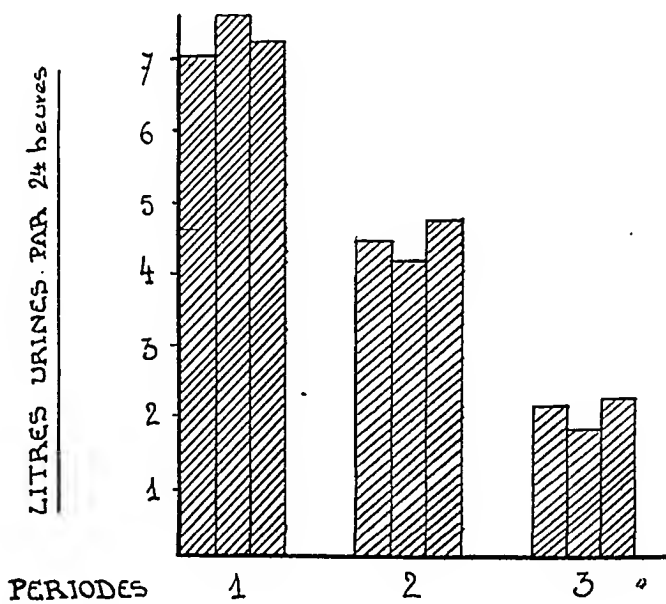
La durée d'action de différentes doses de ce mélange est recherchée. L'administration de 1 ml. de pituitrine à 10 un. par ml. + 1 ml. de polyvinyl-pyrrolidone à 20 p. 100 calme la soif et la diurèse dans 3 expériences successives durant 40 heures, 37 heures et 42 heures. L'administration de 2 ml. de pituitrine à 10 un. par ml. + 2 ml. de polyvinyl-pyrrolidone à 20 p. 100 calme la soif et la diurèse durant 67 heures. L'administration de 3 ml. de pituitrine à 100 un. par ml. + 3 ml. de

polyvinyl-pyrrolidone à 20 p. 100 calme la soif et l'exagération de la diurèse dans 3 expériences successives durant 96 heures, 103 heures et 106 heures.

Depuis 4 mois ce malade se fait tous les 4 jours une injection sous-cutanée de 3 ml. de pituitrine à 10 unités par ml. + 3 ml. d'une solution à 20 p. 100 de polyvinyl-pyrrolidone. Il lui arrive de temps à autre d'éprouver dans les heures qui précèdent l'injection une sensation de soif exagérée et de voir augmenter la diurèse.

Cas 3. — M. Dem. — 18 ans, atteint de diabète insipide depuis 3 mois. La diurèse par 24 heures se situe entre 7 et 8 l. Ni l'examen clinique ni l'examen radiologique, ni les examens de laboratoire ne permettent d'éclairer l'étiologie de l'affection.

Dans une première période, le malade reçoit chaque matin 10 unités de pituitrine. Sous l'effet de ce traitement, la diurèse tombe aux environs de 4,5 l. par 24 heures, mais les in-



Graphique III. — Chaque période indique la diurèse durant 3 jours consécutifs. 1° période: sans traitement, 2° période: 10 unités de pituitrine tous les matins, 3° période: 10 unités de pituitrine + 25 cg. de polyvinyl-pyrrolidone (1 ml.) tous les matins.

jections provoquent de la pâleur, des céphalées et des palpitations, rarement une diarrhée. L'action anti-diurétique des injections de pituitrine ne dure qu'environ 8 heures ce qui n'appaise la sensation de soif que durant une partie seulement de la journée.

Le malade dans une seconde période reçoit chaque matin une piqûre d'un ml. d'un mélange contenant par ml. 10 unités de pituitrine et 25 cg. de polyvinyl-pyrrolidone. L'injection du mélange ne provoque aucun malaise et combat la polyurie et la sensation de soif durant le nycthémère entier; elle ne provoque aucun malaise. La diurèse par 24 heures est d'environ 2 l.

Dans la suite la durée d'action des différentes doses de ce mélange est déterminée. Avec 1 ml. de ce mélange, dans 3 expériences successives le malade voit la sensation de soif et l'exagération de la diurèse calmées durant 37 heures, 32 heures et 39 heures. Avec 2 ml. de ce mélange, dans 3 expériences successives, l'activité se prolonge durant 52 heures, 57 heures et 59 heures. Avec 3 ml. de ce mélange, l'activité se maintient durant 75 heures, 74 heures et 83 heures.

Depuis 4 mois, ce malade s'administre tous les 3 jours une injection de 3 ml. de ce mélange. La diurèse se maintient aux environs de 2 l. et la sensation de soif anormale n'a plus jamais reparu. Il existe durant 3 heures environ un endolorissement au point d'injection, mais il n'y a jamais de malaise d'ordre général.

DISCUSSION

La prolongation de l'action de la pituitrine doit s'expliquer par l'absorption de l'hormone protéinique sur la grosse molécule de polyvinyl-pyrrolidone, ce qui explique aussi l'absence de malaises après les injections.

Claisse, Choay & Choay (1947 a) ont en effet constaté que si à un extrait post-hypophysaire titrant 20 unités ocytociques par ml. on ajoutait 30 p. 100 de polyvinyl-pyrrolidone, ce mélange ne donnait plus au dosage physiologique sur la corne

utérine in vitro qu'un titre de 8 unités ocytociques. La dissimulation de 60 p. 100 des unités ne peut s'expliquer que par une adsorption. Par ailleurs, cette dissimulation ne se produit pas si les deux produits sont amenés successivement au contact de la corne utérine in vitro.

Ces mêmes auteurs ont recherché si l'administration de polyvinyl-pyrrolidone par voie intraveineuse pouvait avoir une influence sur l'action de la pituitrine en injection intramusculaire. Ils ont constaté que l'injection intraveineuse de 40 ml. d'une solution à 20 p. 100 (soit 8 gm. de polyvinyl-pyrrolidone) prolongeait légèrement l'action de la pituitrine mais n'atténuait nullement les malaises provoqués par son administration.

Cette méthode constitue un progrès certain dans le traitement du diabète insipide; en effet, comme nous avons pu le mettre en évidence dans les trois cas où nous avons rapporté le résultat de ce traitement, ce mode d'administration d'une part supprime les malaises provoqués par l'administration séparée de pituitrine et d'autre part calme efficacement la sensation de soif qui dans les cas graves, comme dans le premier cas peut être torturante, sans nécessiter la répétition des piqûres.

On pourrait objecter à cette méthode qu'il est plus simple d'administrer la pituitrine en prise nasale. Le cas 1 montre cependant que la prise nasale peut provoquer des intolérances graves, en l'occurrence un asthme intense. Par ailleurs le peu de durée d'action des prises nasales force les malades à répéter celles-ci nuit et jour, ce qui finit par créer un véritable état d'obsession. Celui-ci est aggravé par le fait de la rareté de la poudre de post-hypophyse qui fait que la recherche de ce produit devient dans certains cas une véritable psychose.

L'administration de doses de l'ordre de 30 unités en injection sous-cutanée, en prolongeant fortement l'action anti-diurétique de la pituitrine permet de réduire au minimum les inconvénients inhérents aux injections sous-cutanées puisque les malades peuvent espacer leurs injections de deux à quatre jours.

Voici un tableau résumant la prolongation de l'effet de la pituitrine associée au polyvinyl-pyrrolidone dans les 3 cas étudiés.

Tableau I.

Prolongation de l'effet de la pituitrine par le polyvinyl-pyrrolidone.
Durée d'activité notée en heures.

	Pituitrine seule 10 unités	Pituitrine associée au polyvinyl-pyrrolidone		
		10 unités	20 unités	30 unités
cas 1	5 h.	28 h.	41 h.	52 h.
	6 h.	31 h.	43 h.	54 h.
		29 h.		49 h.
cas 2	8 h.	40 h.	67 h.	96 h.
	10 h.	37 h.		103 h.
		42 h.		106 h.
cas 3	8 h.	37 h.	52 h.	75 h.
		32 h.	57 h.	74 h.
		39 h.	59 h.	83 h.

Cette méthode rend la vie des malades beaucoup plus confortable. Elle évite les malaises si souvent provoqués par l'injection de pituitrine seule, elle enlève au malade la préoccupation de devoir renouveler les piqûres après quelques heures et leur permet de ne faire une piqûre que tous les deux ou quatre jours. Elle permet en outre une économie importante d'hormone puisqu'elle permet à des malades qui avaient besoin de 20 à 50 unités par jour de ne se faire que 30 unités tous les 2 ou 4 jours.

Claisse, Choay & Choay (1947 b) ont remarqué qu'en cas de fatigues physiques importantes, la durée d'action du médicament était diminuée.

RÉSUMÉ ET CONCLUSION

L'association du polyvinyl-pyrrolidone à la pituitrine apparaît comme un progrès appréciable dans le traitement du diabète insipide. L'adjonction de cette substance entraîne une

notable prolongation de l'action anti-diurétique de la pituitrine; elle permet d'en réduire assez notablement les doses utilisées et d'autre part elle supprime les malaises que provoque chez le malade l'injection de pituitrine seule.

L'emploi de cette méthode dans trois cas de diabète insipide, dont un particulièrement intense (cas 1), a considérablement amélioré les conditions d'existence des malades en leur permettant de ne s'administrer l'hormone que tous les 2 à 4 jours.

SUMMARY

J. Lederer: Treatment of diabetes insipidus by means of a combination of polyvinyl-pyrrolidone and pituitrin.

The addition of polyvinyl-pyrrolidone to posterior lobe extracts (pituitrin) must be regarded as a valuable improvement in the treatment of diabetes insipidus. The advantages of the use of the combined preparations are the following: 1° a marked prolongation of the antidiuretic action of pituitrin; 2° the possibility of using much smaller doses of pituitrin; 3° the elimination of the very disagreeable symptoms usually produced by pituitrin alone.

In three cases of diabetes insipidus, one of which was especially severe (case 1), this method of administration considerably improved the well being of the patients, as the injections could be given every other or every fourth day.

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GROWTH HORMONE AND BLOOD SUGAR LEVEL

BY

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The experiments of *Young* (1945) have shown that injections of growth-promoting hypophyseal extracts may cause diabetes in dogs. The way in which this effect is produced, has not yet been fully elucidated, but it may be due to an overstimulation of the insulin-production by the pancreas, resulting in a lesion of the pancreatic islets. This view is supported by the fact that stimulation of the insulin production by other agents, e. g. by means of continuous or frequently repeated dextrose infusions, also leads to a lesion of the islets (*Woerner*, 1938, and *Dohan et al.*, 1948).

The stimulation of the insulin production might be a direct or an indirect effect of the extract. In the first case the extract would have a »pancreatrophic« effect, in the second case the effect might be due to interference with carbohydrate consumption. Arguments in favour of the second supposition were obtained in experiments which we have recently reported (*Gaarenstroom et al.*, 1949).

On the other hand a pancreatrophic effect may also be involved. An argument in favour of such an action is the very low blood sugar level which follows the single administration of a highly purified growth hormone preparation (*Marx et al.*, 1944). In addition a pancreatrophic effect might be concluded

from experiments with rats hypophysectomized 3—6 weeks previously in which the administration of sugar led to a considerable rise in blood-sugar concentration (*Hublé & Gaarenstroom*, 1947). In 27 operated animals the blood-sugar value reached an average level of 317 mg. per cent, whereas the average of 32 controls was only 129 mg. per cent. Experiments performed with only a small number of rats revealed that correspondingly high values were not obtained in hypophysectomized rats if they were treated with growth-promoting hypophyseal extracts. This suggested that the apparent disturbance of the carbohydrate metabolism in the hypophysectomized rats was due to the absence of the growth-promoting hormone.

Since then, these experiments have been repeated with a larger number of rats. As our growth-hormone preparation*) was but partly purified and contained a. o. substances which are known to exert an effect on the adrenals and on the thyroid, similar experiments were carried out with adrenalectomized-hypophysectomized and thyroidectomized-hypophysectomized animals.

METHODS

Rats varying in weight between 100 and 200 gm. were used. The adrenals were removed one week after hypophysectomy and the thyroid gland three weeks before hypophysectomy. This proved to be the best way of reducing the mortality of these combined operations. The administration of the growth-hormone preparation was begun immediately after the removal of the hypophysis, a dose of 10 R. U. (tail units) being given twice daily. The adrenalectomized rats were maintained by the administration of desoxycorticosterone acetate, of which 0.5 mg. was given daily; this treatment was continued during the whole course of the experiment.

Blood-sugar estimations were carried out on the 14th and 21st day after hypophysectomy, except in the case of the adre-

*) Kindly put at our disposal by Dr. Dingemanse (Amsterdam).

nalectomized animals in which only a single estimation was made on the 21st day after hypophysectomy.

The sugar was administered as a 20 per cent solution of dextrose, of which 3 ml. was usually given orally, followed after one hour by an intraperitoneal injection of 2 ml. In the last series of experiments the whole amount (5 ml.) was given intraperitoneally. One hour after the intraperitoneal injection the blood-sugar value was determined. In the 16 hours preceding the administration of sugar the animals received no food.

RESULTS AND DISCUSSION

Table 1 gives the blood sugar values of all experimental animals. Group A refers to the hypophysectomized rats; group B to the hypophysectomized-adrenalectomized ones and groups C and D to the hypophysectomized-thyroidectomized animals. In each group about half the animals were injected with growth-hormone, whereas the other ones received no further treatment, and served as controls. The figures in the columns marked A_1 , C_1 and D_1 refer to estimations carried out on the 14th day after hypophysectomy, the figures in the columns A_2 , B , C_2 and D_2 to estimations carried out on the 21st day after the operation. Therefore there are in these groups, as a rule, for each rat two figures, which are to be found on the same line. The first figure in the first column and the first figure in the third column belong to the same rat; similarly the seventh figure of the twelfth and of the fourteenth column, etc.

We have not found in our present control animals the high blood sugar values of 300 mg. per cent or more which were previously obtained except in the animals which were hypophysectomized and thyroidectomized. The average value, though being appreciably higher than in normal rats treated in the same way, remains in all cases below 200 mg. per cent. The reason for the discrepancy between the actual values obtained in these and in the previous experiments is unknown.

In group A the average blood-sugar value of the treated animals on the 14th day after hypophysectomy was found to

Table 1.
Blood sugar values in mg. per 100 ml.

A ₁		A ₂		B		C ₁		C ₂		D ₁		D ₂	
hypo- physectomized				hypophy- sectomized + adrena- lectomized		hypophysectomized + thyroidectomized							
gr. h.	contr.	gr. h.	contr.	gr. h.	contr.	gr. h.	contr.	gr. h.	contr.	gr. h.	contr.	gr. h.	contr.
100	53	105	166	85	105	102	240	†	†	78	310	470	527
101	112	170	122	93	113	107	314	474	†	117	351	147	†
106	116	96	121	160	129	114	340	220	†	131	414	367	†
110	151	106	347	112	130	156	371	483	†	196	421	474	†
114	161	100	227	118	150	163	392	†	†	389	421	431	†
120	196	122	128	149	156	221	406	255	†	397	432	385	136
141	200	108	132	151	206	278	430	348	†	411	443	385	†
164	234	116	318	156	209	371	512	†	†	413	488	140	†
183	244	180	†	237	476			†	352	432	528	451	†
		72	79	251					492	453	517	402	†
		72	96	251					508	461	619	293	700
		75	102	310						461		468	†
		83	118										
		93	132										
		93	170										
		93											
		100											
		109											
123	163	105	161	121	187	221	376			329	453	368	455
1	1.3	1	2.4	1	3.2	1	2.9			1	2.4		

* † means that the animal had died.

be 123 (A₁) and on the 21st day 105 mg. per cent (A₂), which means that it had remained on the level which is normal for non-hypophysectomized rats. Because of the rather low blood-sugar values found in the controls and the wide range of variability, the difference between the injected animals and the controls was not significant. Similar differences were, however, found in all the other groups of experiments, this suggests that the differences between the results found in the treated and control animals are real also at least in group A₂.

If an effect of the pituitary extract on the blood-sugar level is accepted, it will be necessary to find out whether this effect

is due to the presence of hormones other than the growth hormone in the preparation, for example adrenocorticotrophic or thyrotrophic hormone. The figures of columns B, C₁ and C₂ seem to indicate that this is not so. In the rats of group B, which in addition to the hypophysectomy had been adrenalectomized, the differences in blood-sugar level between the experimental animals and the controls are the same as in the rats that had been hypophysectomized only; the same also applies to the hypophysectomized thyroidectomized rats of group C₁ and presumably too to those of group C₂.

We can therefore conclude that treatment with a pituitary extract having a marked growth-promoting activity lowers the increase of the blood-sugar value after sugar administration, and that this effect is not mediated either by the adrenals or by the thyroid, and is therefore probably due to the growth hormone contained in the extract.

It may be that the improvement of the general condition caused by the administration of growth-hormone allows of a more rapid and more efficient use of the available sugar, resulting in a lower blood-sugar value of the treated animals after loading. That the general condition may be of importance, is suggested by the fact that the blood-sugar of animals, which died between the 14th and 21st day of the experiments, was above the average. A poor state of health might diminish the rate of absorption of the sugar given orally. To test this possibility, we carried out a series of experiments in which hypophysectomized-thyroidectomized rats received the whole amount of sugar by the intraperitoneal route (D₁ and D₂). As the difference remained of the same order of magnitude, the influence of the rate of absorption, if present at all, does not seem to be of much importance.

If it can be accepted that the effect of growth hormone is specific, then it is most likely due to a pancreotrophic action. The existence of a »pancreotrophic hormone« has already been a point of debate for years. *Anselmino et al.* (1933) introduced the idea of its existence because they found that the administration of pituitary extracts led to an enlargement

of the pancreatic islets. The same effect was also noted by *Richardson & Young* (1937) and by *Best et. al.* (1942). Removal of the hypophysis, on the other hand, does not lead to atrophy of the islets (cf. e. g. *Houssay*, 1942), and oxidation of sugar continues, although, as our experiments suggest, at a reduced rate.

Although the results of our experiments do not give a decisive answer to the question of the existence or non-existence of a pancreotrophic hormone, they nevertheless seem to be in favour of the idea that growth hormone might possess pancreotrophic properties. In an attempt to offer a physiological basis for the diabetogenic effect of growth promoting hypophyseal extracts we previously suggested that the accumulation of sugar caused by the growth hormone was needed to stimulate insulin production indirectly. Insulin is known to contribute to protein synthesis (*Mirsky*, 1938) and, therefore, may be in some way involved in the process of growth (*Gaarenstroom et al.*, 1949). The indirect stimulus would no longer be necessary if insulin secretion is accounted for by a pancreotrophic influence of the growth hormone. The remaining reason for the diabetogenic influence would then be that it protects the body against hypoglycemia, which otherwise would result from the increased insulin level in the blood.

Further study will be required to elucidate the true nature of this complicated mechanism.

SUMMARY

The increased rise of the blood-sugar level after the administration of sugar in hypophysectomized rats, as compared with normal rats, is prevented by the administration of a growth-promoting pituitary preparation.

Similar or comparable differences between treated and non-treated animals were observed in hypophysectomized-adrenalectomized and hypophysectomized-thyroidectomized rats.

The possibility that the effect of the pituitary preparation might be considered as »pancreotrophic« is discussed.

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THE ELECTROCARDIOGRAM IN HYPOCALCEMIA AND HYPOTHYREOSIS

BY

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In hypocalcemia a prolonged Q—T interval is pathognomonic. In addition, abnormal T-waves are not infrequently found. As a rule they are either low or iso-electric. Occasionally, they may also be negative. Otherwise the tracings do not show any changes which can be regarded as typical of hypocalcemia. In a recent paper dealing with the electrocardiogram (ecg) in hypocalcemia (*Ljung, 1949*), I drew attention to the fact that hypocalcemia alone is probably responsible for the changes in the ecg, particularly in idiopathic and post-operative hypocalcemia (i. e. in hypoparathyroidism). In hypocalcemia due, for instance, to uremia, coma hepaticum, intestinal diseases, etc., other factors may be involved in producing these changes. Among the cases discussed in the paper mentioned above there were twenty cases of postoperative hypocalcemia. In three of them, hypothyreosis was also present. Data on the frequency with which this combination occurs are not available. The number of cases studied was not sufficiently large to allow of any definite conclusions. Nevertheless, the assumption seems to be justified that the combination of post-operative hypocalcemia and hypothyreosis is not uncommon.

In hypothyreosis the T-waves are often low, iso-electric or

negative; occasionally they may even assume a »coronary« shape. In addition, low P-waves and QRS-complexes are fairly often found. Also bundle branch block may occasionally occur. *Zondek* (1918) was the first to draw attention to the changes in the eegs of patients suffering from hypothyreosis. Later, several investigators (*Willius & Haines*, 1925, *Ohler & Abramson*, 1934, and others), confirmed this observation.

From the above statements it is apparent that the abnormal T-waves observed in hypocalcemia and hypothyreosis may have the same configuration. Their behaviour, however, seems to differ. In hypothyreosis abnormal conditions are often revealed by the eeg after exercise or in induced hypoxemia. This also applies to the cases of hypothyreosis in which the eegs at rest are normal (*Larsen*, 1938). In cases of hypocalcemia, the response of the patients to these tolerance tests has hitherto not been studied. In 5 cases of hypocalcemia in which abnormal T-waves were recorded I have personally examined the patients after exercise. All of them responded normally (*Ljung*, 1949).

In some cases of hypocalcemia I observed a marked instability of the T-waves. Normal T-waves alternated with abnormal ones, not only from day to day, but even from hour to hour (*Ljung*, 1949). To my knowledge there are no data available which show that the T-wave also tends to show a similar instability in hypothyreosis. This question requires further investigation.

The main purpose of this paper is to direct attention to the possible existence of a combination of hypocalcemia and hypothyreosis as well as to its significance in the interpretation of the eegs.

MATERIAL AND RESULTS

In the three cases presented here the patients were women on whom subtotal thyroidectomy had been performed for thyreotoxicosis. After this operation the patients developed tetany and myxoedema. A short account of the history of these patients is given in Table 1.

Table 4.

Electrocardiographic changes in response to vitamin D and thyroid therapy in 3 cases of hypocalcemia associated with myxoedema.

No.	Initials	Age	Date	Serum calcium mg. %	R-R interval in sec.	QRS-complexes in mm. ¹)		T-waves in mm. ¹)		Q-T interval in sec.		Therapy	Remarks
						Lead 1	Lead 2	Lead 1	Lead 2	Absolute	Relative ²)		
1	E.J.	56	6.10.44		0.55			1.0	2.0	0.30	+ 0.01		Thyreotoxicosis
			9.5.45									Thyroidectomy	
			16.6.45	5.5-6.3	0.70			1.0	2.0	0.44	+ 0.12	A.T. 10	Tetany
			26.6.45	7.0	0.65			1.0	1.5	0.44	+ 0.13	"	Taken at 8.30 a.m.
			"					0.5	0.5				Taken at 9.30 a.m.
			14.6.46	6.1	0.60			negative	negative	0.47	+ 0.17	Vitamin D ₂ and thyroid	Tetany. Myxoedema
			3.7.46	10.8	0.70			negative	negative	0.41	+ 0.09	"	Myxoedema
			16.7.46	9.8	0.70			0.5	low diphasic			"	
			31.10.46	10.0	0.80			1.0	1.0	0.42	+ 0.08	"	
			21.12.46	9.8	0.70			1.5	2.5	0.36	+ 0.04	"	No symptoms of myxoedema
			30.12.47	15.0	0.60			1.5	2.5	0.34	+ 0.04	Thyroid	"

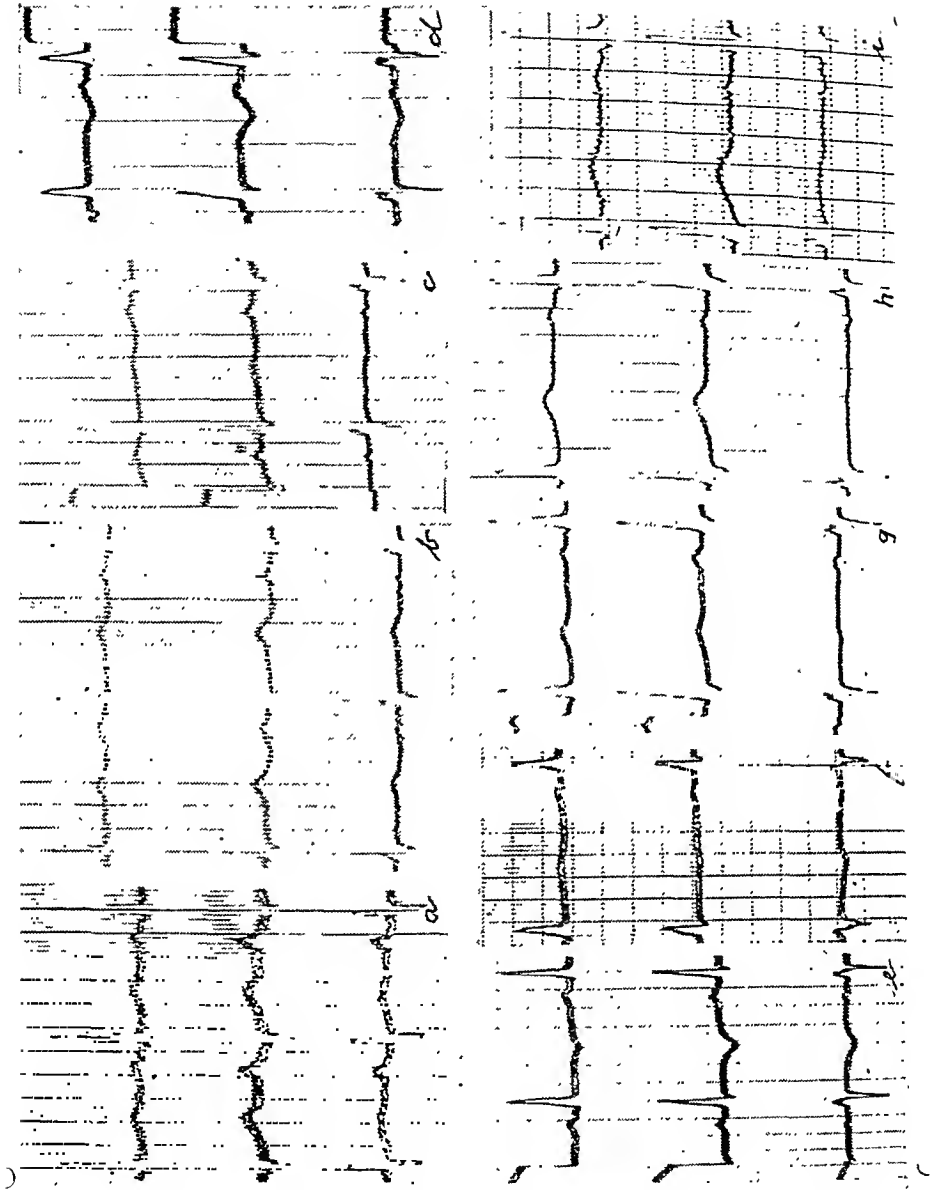


Fig. 1.

Case 1. Electrocardiographic changes correlated with the clinical manifestations.

- a) May 5, 1945: Mild thyrotoxicosis.
- b—c) June 26, 1945: Hypocalcemia following subtotal thyroidectomy. Note changes in the T-waves occurring during the course of one day.
- d) June 16, 1946: Hypocalcemia and hypothyreosis. Negative T-waves and markedly prolonged Q—T interval.

DISCUSSION

In hypocalcemia the serum calcium is usually restored to normal in response to adequate vitamin D₂ therapy within one or two weeks. As the serum calcium rises the ecg usually returns to normal. Hypothyreosis, however, does not respond as readily to routine thyroid therapy. It also takes a much longer time for the ecgs to become normal in these cases.

In case 1, marked changes in the Q—T interval and the T-wave were observed whilst the patient was under observation. The factors involved in producing these changes in this case could be deduced from the general clinical picture. After operation the patient developed hypocalcemia producing prolongation of the Q—T interval. In addition, the T-wave showed a marked instability: normal T-waves alternated with abnormally low ones in the course of one day. After some time, the patient again showed symptoms of tetany. Apart from a marked prolongation of the Q—T interval there was also evidence of negative T-waves in the three usual limb leads. This ecg suggested that hypothyreosis had developed. The other clinical features supported this assumption.

In response to vitamin D₂ therapy combined with thyroid therapy, the serum calcium became normal. This produced only a shortening of the Q—T interval while the negative T-waves persisted. As the hypothyreosis subsided, the T-waves gradually returned to normal. After the condition had been completely cleared up, the T-waves became normal.

Text to Fig. 1 (continued).

- e) July 3, 1946: Normal serum calcium values; hypothyreosis practically unchanged. Q—T interval shorter (compare with d).
- f—g) July 16—Oct. 10, 1946: Normal serum calcium values; hypothyreosis regressing in response to thyroid therapy. T-waves returning toward normal.
- h) Hypothyreosis overcome; serum calcium value restored to normal. Normal tracings.
- i) No evidence of hypothyreosis; hyperealcemia (15 mg. per cent). Broad and rounded T-waves.

For a short period of time the patient exhibited hypercalcemia which produced a typical »rounded« configuration of the T-wave. There were no appreciable changes in the QRS-complex in this case whilst the patient was under observation.

In case 2 the serum calcium was restored to normal after ten days' treatment with vitamin D₂ and thyroid. The T-waves which were abnormally low, increased only very slightly during the course of this therapy. The value of their height corresponded at that time to that usually considered as representing the limit between a normal and an abnormal T-wave. The QRS-complex which was at first rather low, did not show any changes. After treatment had been continued for another few weeks, the myxoedema was cleared up and both the T wave and the QRS-complex increased markedly in height. In this case too hypercalcemia was found on one occasion. As in case 1 the T-wave assumed a typical »rounded« shape.

In case 3 the patient was at first treated only with vitamin D₂. After ten days of this therapy the serum calcium was restored to normal. The abnormally low T-waves increased slightly in height but they did not return to normal. In response to thyroid therapy, however, the hypothyreosis gradually regressed, the T-waves became normal, and the QRS-complex increased markedly in height.

In the same way, as marked hypocalcemia may be present in the absence of manifest tetany, hypothyreosis may be present in the absence of myxoedema. Nevertheless, both these conditions may produce changes in the eeg. If there is a combination of hypocalcemia and hypothyreosis, either condition may be »asymptomatic« and may escape observation. If this happens, the condition producing typical symptoms may incorrectly be made responsible for the electrocardiographic changes seen in a given case. The relatively low QRS-complexes observed in cases 2 and 3 were produced by hypothyreosis. Although hypothyreosis seems to have been a contributory factor in producing the prolongation of the Q—T interval, these two cases suggested that hypocalcemia was the

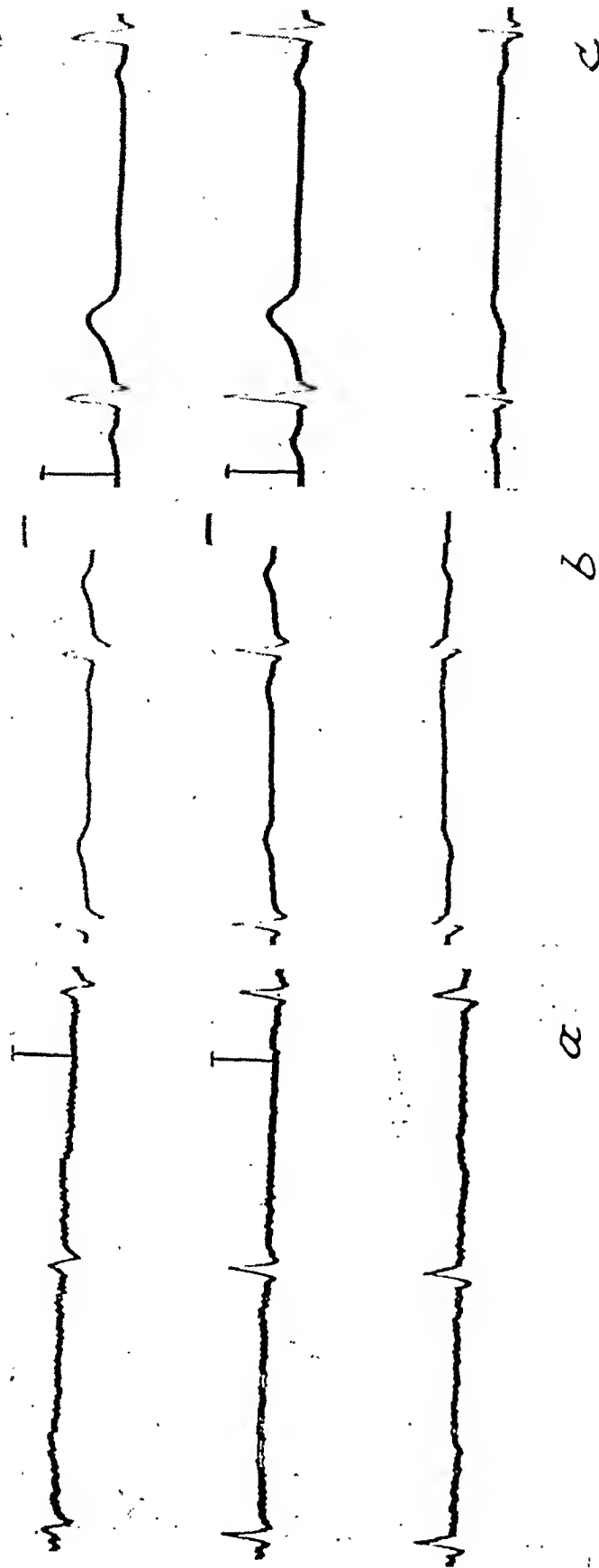


Fig. 2.

a) Sept. 19, 1946: Hypocalcemia and hypothyreosis. Abnormally low T-waves.

b) Sept. 28, 1946: Serum calcium value restored to normal in response to thyroid and vitamin D therapy; myxoedema persisting.

c) Nov. 4, 1946: Serum calcium value restored to normal; hypothyreosis overcome. Normal T-waves. QRS-complexes have markedly increased.

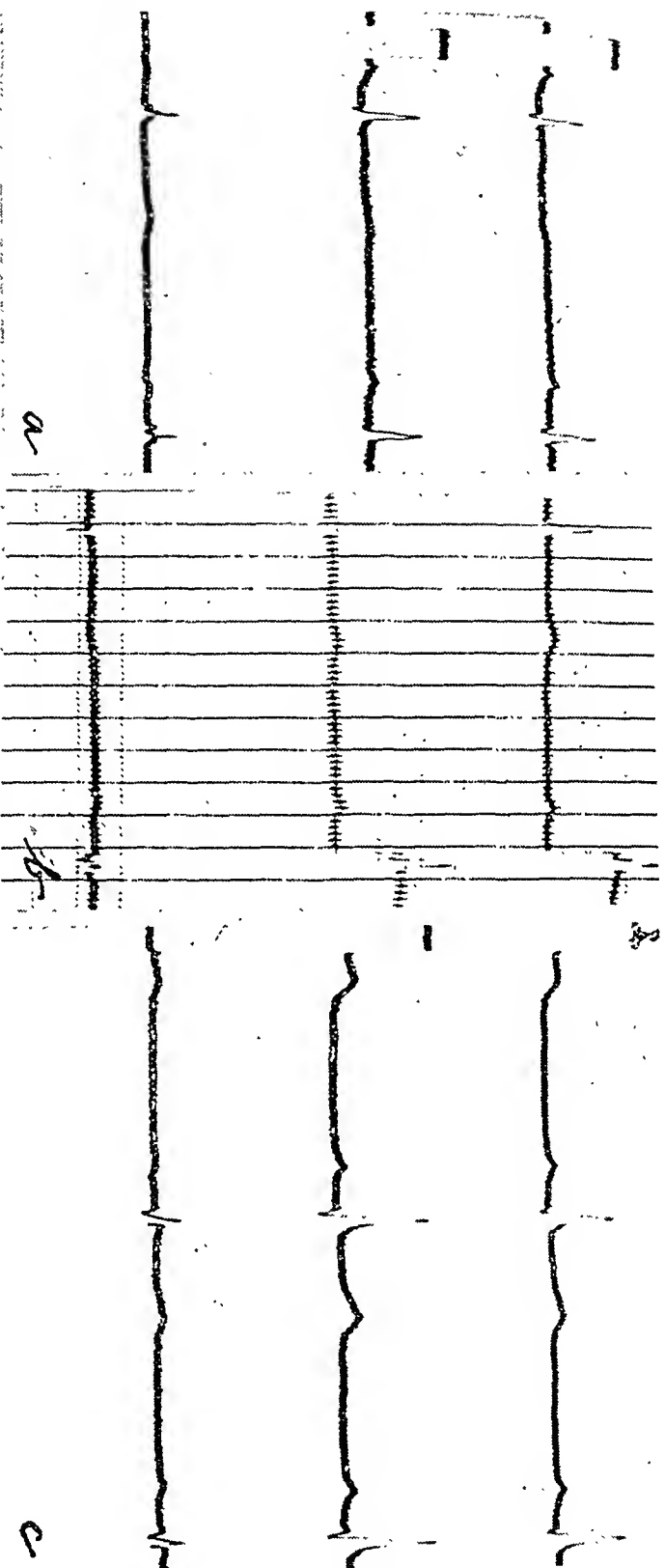


Fig. 3.

Case 3. Electrocardiographic changes correlated with the clinical manifestations.

- a) March 5, 1946: Hypothyreosis and hypocalcemia. Abnormally low T-waves.
- b) March, 16, 1946: Serum calcium value restored to normal in response to vitamin D therapy. T-waves have slightly increased in height. Q-T interval shorter. QRS-complexes practically unchanged.
- c) May 3, 1946: Following thyroid therapy. T-waves became normal and the QRS-complexes markedly increased in height.

main factor involved. It may seem strange that on the occasion when abnormally high serum calcium values were recorded in cases 1 and 2, the length of the Q—T interval corresponded to that found in the presence of normal serum calcium values. In cases of hypercalcemia I have often observed that the T-wave assumes a more »rounded« shape than in the presence of a normal calcium value, whilst the Q—T interval does not undergo any appreciable changes. This also applies to cases 1 and 2 discussed here. This phenomenon which has to my knowledge not yet been described in the literature, will not be discussed here at any length. At the present stage of our knowledge it is not possible to determine its significance.

If there is a combination of hypocalcemia and hypothyreosis, either condition may be the main factor responsible for producing the low and negative T-waves. In the three cases presented here hypothyreosis was the main contributory factor. The abnormal T-wave did not show any instability. Nor did subcutaneous or intravenous administration of 1/4 mg. of ergotamine (Gynergen-Sandoz) bring about any appreciable changes in the T-waves.

In cases in which hypocalcemia or hypothyreosis are not recognized because of the absence of typical signs or symptoms the abnormal tracings are, as a rule, incorrectly ascribed to an organic myocardial lesion. This applies in particular to the abnormal T-waves. The factors producing them in these cases are still undetermined. It is generally assumed, however, that they are not due to an organic myocardial lesion but rather to a functional disturbance.

SUMMARY

The shape of the electrocardiogram in hypocalcemia and hypothyreosis is briefly discussed. It is pointed out that the eeg may be abnormal in hypocalcemia without tetany, as well as in hypothyreosis without myxoedema. If these conditions are not recognized, the electrocardiographic changes may incorrectly be ascribed to an organic myocardial lesion which probably does not exist.

Three cases of manifest tetany associated with myxoedema are reported. These cases were observed among 20 cases of postoperative hypocalcemia discussed in a previous paper. On the basis of the general clinical picture it was possible in these cases to differentiate between the electrocardiographic changes due to hypocalcemia and those produced by hypothyreosis. It is emphasized that the possible presence of a combination of hypocalcemia and hypothyreosis should always be considered. Otherwise it is possible that the changes in the electrocardiogram which are due to hypothyreosis, may be incorrectly ascribed to hypocalcemia or vice versa. Moreover, the possibility should always be borne in mind that the changes may be due to both conditions.

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ABRUPTIO PLACENTAE IN VITAMIN E DEFICIENT GUINEA PIGS*)

BY

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In a series of papers *E. V. Shute* (1935—1942) states that a deficiency of vitamin E can induce premature separation of the normally implanted placenta in women, and that vitamin E therapy can cure the disease if given at an early stage. He supports his theory by clinical experience, and by experiments in animals using his reaction for the estimation of free oestrogenic substances in blood. The anti-proteolytic properties of blood serum are determined by trypsin digestion, and he claims that the digestion is inhibited by an oestrogen. In cases of abruptio placentae the blood level of this substance is raised. This fact should, according to *Shute*, disturb the normal proteolytic implantation of the placental villi, and thus cause the premature separation of the placenta. However, objections have been raised against *Shute's* reaction (*Cuthbertson & Drummond*, 1939; *Drummond, Noble & Wright*, 1939).

In order to study this problem a series of experimental investigations have been made, the first of which is presented here. Guinea pigs were chosen as experimental animals as they have a hemochorial placenta like women. In this species, experimental separation of the placenta has been produced by

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giving the pregnant animal injections of histamine (*Hofbauer*, 1926), oestrogens or gonadotrophins (*Zondek*, 1929; *Kelly*, 1931). The gonadotrophins have been thought to cause the premature detachment by stimulating the ovaries to increased oestrogen production.

In experiments on five guinea pigs *Pappenheimer & Goettsch* (1941) observed death of the fetus in pregnant animals on a diet low in vitamin E. In spite of the fact that the diet was supplemented with 25 gm. of fresh lettuce daily, and that each animal received 5—10 mg. of alpha-tocopherol acetate each week haemorrhagic and anaemic necrosis of the placentae developed. The authors state that a blood stained vaginal discharge was observed, but do not mention whether a premature separation of the placentae was observed, nor do they describe any pathological changes in the placental vessels. Examination of the dead fetuses did not give any clear explanation of the cause of death. They stated that further observations were necessary before one could conclude that the placental lesions were of primary importance. I have been unable to find any further studies on this problem in the available literature.

MATERIAL AND METHODS

Female guinea pigs were used, weighing 200—300 gm. at the beginning of the experiments. They were all from the same stock and were obtained through a local dealer. They ate a diet of casein-dextrin type, Diet 12 (*Ingelman-Sundberg*, 1948), i. e. almost free of vitamin E, as all constituents except the lard (6 per cent of dry substance) had been extracted with alcohol and ether. All animals were given the diet for at least three months before they were mated (body weight about 500 gm.). Five animals were given Diet 12 only plus 2.5 mg. ascorbic acid daily (group B). Four guinea pigs received the same diet, but during pregnancy vitamin E was injected intraperitoneally on different days of pregnancy as an aqueous solution of dl-alpha-tocopherylphosphate ($C_{29}H_{49}O_2 \cdot PO_3Na_2$) (group P). Sixteen animals were each given Diet 12 supple-

mented with 2.5 mg. ascorbic acid and 0.04—1.60 mg. alpha-tocopherol daily from the beginning of the experiment (groups C—O). Twelve animals receiving hay, turnips etc. served as controls (group A). The vitamin E was given by tube in the pharynx daily as an aqueous solution of alpha-tocopherylphosphate. For details of feeding, a previous article should be consulted (*Ingelman-Sundberg, 1948*). The animals were mated in proestrus by being placed with a normal male in a cage for 24 hours. In this way the exact date of conception could be fixed. After mating the vaginal membrane was observed every day. When the vagina was open, and a blood stained discharge was seen, this was always taken as a sign of beginning separation of some of the placentae. The animals were then killed and examined. In most cases the placentae were at any rate attached to the uterine wall, which then was also removed for histological examination. The placentae were fixed in 10 per cent formalin, and after gelatine embedding were cut in 10 μ sections with a freezing microtome. Paraffin sections of 5 μ were also prepared. Unstained frozen sections were mounted in glycerol for polarizing microscopy. Other sections were stained with haematoxylin and eosin or haematoxylin and van Gieson's stain. Specimens were also taken from the extensor musculature of the thighs and fixed in sublimate formalin (concentrated solution of mercuric chloride 1 part, 35 per cent formalin 1 part, distilled water 2 parts) stabilized with excess of iodine (*Ingelman-Sundberg, 1934*). Paraffin sections of 5 μ were stained with haematoxylin and eosin or with haematoxylin and van Gieson's stain. The control animals were killed on different days of pregnancy, and the placentae were examined in the same manner as the other placentae. No specimens, however, were taken from the musculature in these animals.

RESULTS

The results are presented in Table 1. Only the controls and the animals receiving at least 1.60 mg. free tocopherol daily continued to full term. The other animals all showed a total or partial premature separation of the placentae.

In all cases except animal N 6 the fetuses were normal. In this case one fetus was a monster, but the other one was normal. The fetuses of animals A 48 and B 24 were examined histologically, and a large number of sections made, but no pathological changes were detected. Animal F 30 gave birth to a fetus on the 63rd day of pregnancy. It lived for only 3 hours, but at autopsy was found to be normal, and the histological examination of the musculature did not reveal any pathological changes. Animal N 8, which was killed on the 60th day of pregnancy showed no signs of placental separation. Three living fetuses were found at the time of autopsy, but only one of them remained alive; it lived for three hours in spite of the fact that no animal could take care of it. In the thigh musculature of this particular fetus some microscopic haemorrhages were seen, but no muscular dystrophy was observed. Animal P 36, which had received 12 mg. of free tocopherol on the 20th, 45th, and 55th days of pregnancy had a premature separation of the placenta on the 60th day, when it was killed. Two normal fetuses were found. One was asphyxiated and did not breathe despite normal heart action; the other one lived for two hours. Microscopical examination showed that the musculature was normal in both. Premature guinea pigs seldom remain alive, even if the prematurity only amounts to 3—4 days. Thus it is quite reasonable to expect that guinea pigs born on the 60th to 62nd day instead of on the normal 68th to 70th day should die shortly after birth.

The different placentae of any one animal always showed the same picture even if the severity of the changes varied to some extent. Table 1, therefore, shows the mean changes

Text to Table 1.

Observations on normal pregnant guinea pigs (*series A*), pregnant guinea pigs given an almost vitamin E free diet for at least 3 months before mating (*series B*), pregnant guinea pigs on the same diet supplemented by tocopherol 0.04—1.60 mg/day (*series C—O*), and on E-deficient pregnant guinea pigs given vitamin E by injection on different days of pregnancy (*series P*).

Animal	Tocopherol mg/day	- Abortion day and day of delivery (a) Execution day (+)											Muscu- lar dys- trophy	Placenta		Birefringent microcrystal- line substance	
		15	20	25	30	35	40	45	50	55	60	65	70	Vascular changes	Large bire- fringent crystals	Placenta	Uterine wall
A 12	-																
20	-																
22	-																
32	-																
35	-																
37	-																
48	-																
51	-																
52	-																
56	-																
57	-																
65	-																
B 16	0																
17	0																
22	0																
24	0																
31	0																
C 16	0.04																
17	0.04																
20	0.04																
D 12	0.08																
14	0.03																
E 14	0.20																
F 10	0.40																
20	0.40																
24	0.40																
30	0.40																
G 6	1.20																
8	1.20																
O 1	1.60																
3	1.50																
5	1.50																
8	1.60																
P 18	0																
28	0																
36	0																
38	0																

Table 1.

found in the placenta of each animal. Placentae older than 55 days did not show any pathological changes, except those characteristic of the normal ageing placenta. In placentae which had separated earlier than the 55th day, marked pathological changes were often found. On gross examination, com-



Fig. 1.

The uterus of the E-deficient guinea pig P 18 (Table 1), which died on the 45th day of pregnancy due to partial separation of the 2 placentae to the left, followed by a large intrauterine haemorrhage. The placenta to the right does not show any separation, but as in the other placentae necrotic areas and haemorrhages are seen.

Fixation in 10 per cent formalin.

plete or partial separation of at least one placenta was found with placental haematomata or haemorrhages outside the fetal membranes, and areas of anoxic and haemorrhagic infarction were regularly present (Fig. 1). In animal P 18 the intrauterine haemorrhage was so great that it caused the sudden death of the mother on the 45th day of pregnancy. On microscopical examination, degenerative vascular changes of the maternal vessels of the placenta were seen; this probably being the cause of the necrosis and the haemorrhages (Figs. 2—4).

In the large and medium sized vessels they chiefly consisted of papillomatous proliferations of the intima and incrusted deposits in the other layers of the vessel, where atypical syncytial giant cells were seen. The changes in the small vessels somewhat resembled those of a vasculitis, and there was in-



Fig. 2.

Section from the middle placenta of Fig. 1. Haematoxylin and van Gieson's stain. Magn. 6 X. A haematoma is observed in the middle of the picture, and large necrotic areas are seen.

filtration of leucocytes and penetration of granulation tissue, thrombosis also being occasionally present. In some respects the changes found resemble the arteriosclerosis of the spiral arterioles of human placentae in toxæmic premature separation (Hellman, 1947).

On examining the frozen sections of the placentae and uterine walls a birefringent substance, as described by *Dempsey & Wislocki* (1944), was seen (Fig. 5). The amount of this substance varied, and bore no significant relation to the amount of vitamin E supplied to the animal (Table 1).



Fig. 3.

Section from the right placenta of Fig. 1. Haematoxylin and van Gieson's stain. Magn. 24 \times . In the upper part an infarction is seen. In the intima of the vessel below, papillomatous proliferations are present, and atypical, syncytial giant cells are observed in its outer layers to the right.

In several of E-deficient placentae, large, sudanophil, birefringent crystals of a yellow colour were found near the base of the placenta (Fig. 5). They did not stain with iodine, nor was there any discoloration with 1 per cent chromic acid,

and the iron reaction was negative. The Liebermann-Burchard reaction by the Schultz method was negative, and a brown-red colour appeared after treatment with sulphuric acid. Because of these various reactions, the crystals were considered to be some kind of metabolic pigment.

In animals P 36 and P 38, which after mating had received larger amounts of vitamin E by injection, neither vascular changes nor crystals were seen. In animal P 28, which received 24 mg. vitamin E on the 28th day of pregnancy, there were



Fig. 4.

Section from the labyrinthine zone of a placenta, which has not yet separated from the E-deficient animal B 24 (Table 1). Haematoxylin and van Gieson's stain. Magn. 24 X. Four circumscribed areas of infarction are observed, one in the upper left corner of the picture, the other smaller ones, to the right below.

some vascular changes but no crystals at the time of placental separation on the 40th day. In animal P 18, however, which had received 12 mg. tocopherol on the 28th day both were present at the time of separation on the 45th day. The placenta of animal C 17, which was examined on the 15th day of pregnancy, showed neither vascular changes nor crystals. In animal C 20, in which separation occurred on the 20th day there were vascular changes but no crystals, and similar findings were made in animals C 16, D 14, E 14, and F 20.



Fig. 5.

Unstained section of a placenta and the corresponding part of the uterine wall of the E-deficient guinea pig B 16 (Table 1). Crossed Nicols. $\times 5$ oc., $\times 3.2$ obj. A microcrystalline, birefringent substance is faintly visible towards the base of the picture corresponding to the labyrinth, and in the upper part of the picture corresponding to the uterine wall. In between, large, double refracting crystals are seen lying in the subplacenta.

DISCUSSION

From the results it is evident that the premature separation of the placentae was caused by an inadequate supply of vitamin E, as only the controls and the experimental animals receiving 1.60 mg. of free tocopherol were able to carry their young to term. As seen from Table 1, the separation of the placentae also took place at a later period of pregnancy when increasing amounts of tocopherol were administered orally or given by injection.

Examination of the placentae separated prematurely revealed severe vascular changes in the maternal vessels in-

volving infarction of the placental tissue and haemorrhages. Such changes were not found among the controls nor when sufficient amounts of vitamin E were given. Hence a hypovitaminosis seems to be the primary cause of these changes.

In several prematurely separated placentae crystals consisting of some sort of metabolic pigment were found near the attachment of the placenta to the uterine wall. These crystals were never seen without accompanying vascular changes, but less severe vascular changes were found in the absence of any crystals. These may therefore possibly be a product formed during metabolic changes in infarcted tissues.

Similar placental alterations have been described by *Sammartino & Blanchard* (1946) in rabbits receiving oestrogens during pregnancy. In placental separations caused by histamine, as described by *Hofbauer* (1926), however, no comparable vascular changes were found. Judging from the pathological findings, therefore, an oestrogenic effect may be the cause. An investigation of this problem is now in progress. Before these latter experiments are completed, no definite conclusions can be drawn about the results obtained by *Shute*.

SUMMARY

Guinea pigs were given an almost vitamin E free diet, and varying amounts of dl-alpha-tocopherylphosphate. After three months the animals were mated.

With a daily supply of less than 0.4 mg. free tocopherol the animals aborted on the 20th to 30th day of pregnancy due to necrosis and premature separation of the placentae. With increasing amounts of vitamin E abortions took place at successively later stages of pregnancy, and the pregnancy ended with normal delivery when the animal received 1.60 mg. daily.

The separation of the placentae was caused by changes in the maternal vessels of the placenta, and there was infarction of the placental tissue and haemorrhages. These changes were often followed by the deposition of large, birefringent, yellow crystals consisting of a metabolic type of pigment. All these

pathological changes were prevented by a sufficient supply of vitamin E. The origin of the changes is discussed.

ACKNOWLEDGEMENTS

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THE ASCORBIC ACID CONCENTRATION IN THE BLOOD OF THE RABBIT

CHANGES UNDER NORMAL CONDITIONS, FASTING AND
AFTER STIMULATION WITH GONADOTROPHIC
HORMONES

BY

BERNT HÖKFELT

Andrews and co-workers have studied the effect of gonadotrophic stimulation on the ascorbic acid concentration in the blood of various animals. In bovine animals they demonstrated a considerable and relatively rapid decrease in the ascorbic acid content following the administration of pregnant mare serum gonadotrophin (PMS) (*Erb & Andrews, 1942, Andrews & Erb, 1942*). No such change, however, seemed to occur in the chicken and the rat (*Andrews & Erb, 1943, Almquist & Andrews, 1944*). *Lardy, Casida & Phillips (1944)* observed a decrease in the blood ascorbic acid level after a first injection of pregnant mare serum (PMS) in the sheep; a further injection, however, caused a rise. The same authors demonstrated a decrease in the content of ascorbic acid in the blood after the injection of PMS both in pseudopregnant and in anoestrous rabbits.

The effect of gonadotrophic stimulation thus appears to vary in different species of animals. Routine determinations of the blood ascorbic acid of the rabbit performed in connec-

tion with other investigations on the effect of the gonadotrophic hormone have given results indicating that gonadotrophic stimulations is not the only cause of the variations. The present investigations were made on the changes in ascorbic acid content of the blood of the rabbit; its normal variations during 24 hours fasting, as well as those observed after the administration of pregnant mare serum gonadotrophin were determined. In order to exclude the possibility that the serum gonadotrophin would have a secondary effect on the ascorbic acid in the blood through the formation of oestrogenic hormones by the ovary, experiments were also made on spayed rabbits.

MATERIAL AND METHODS

The experiments were performed on adult female rabbits only. *Determination of the blood ascorbic acid:* Blood was withdrawn by means of arterial puncture of the ear. The wound was heparinized and closed with a clamp after each sample was taken. Only in exceptional cases was it necessary to make more than one puncture. 5 ml. of blood was precipitated with 15 ml. of 6 per cent trichloroacetic acid. After filtration the samples were treated with norit. Determination of the ascorbic acid concentration was then made immediately, using the method described by *Roe, Kuether et al.* with 2,4-dinitrophenylhydrazine (*Roe & Kuether, 1943, Roe & Oesterling, 1944, Mills & Roe, 1947*). The colorimetric determinations were made with a Beckman spectrophotometer (wavelength 540 m μ).

Pregnant mare serum gonadotrophin (Antex Leo¹) was administered intravenously in a dose of 450 I. U.

The anaesthetic used in ovariectomy was citodon-Na (Leo¹).

The diet consisted of turnips, hay and grain; the animals were fed every day at approximately 10 a. m. The fasting animals were allowed free access to water.

¹) These preparations were kindly placed at the writer's disposal by A.-B. Leo, Hälsingborg.

RESULTS

Table 1 shows the ascorbic acid concentration in the blood of normal rabbits at different times during the 24 hours. The concentration at 12 noon was chosen as the basic value. This varied in the different animals within the limits of 0.92 to 1.38 mg. per cent. A gradual increase took place during the ensuing hours. A maximum was reached at midnight with an average increase of 47 per cent. The concentration then started to fall and at 9 a. m. was 14 per cent below the basic value. After 24 hours the concentration had once more risen to its original level.

As can be seen from Table 2, fasting for 30 hours caused a decrease of 44 per cent as compared with the ascorbic acid concentration found at 12 noon on the day before the beginning of the fasting period. After a further six hours' fasting, the level fell by approximately 60 per cent and thereafter during the following 18 hours remained relatively unchanged at this low level.

The changes in the ascorbic acid concentration following an intravenous injection of 450 I. U. PMS at 12 noon were studied in a third group of animals. A comparison of Tables 1 and 3 reveals that there was no appreciable difference between the 24-hour variations in this group and in those animals to which no gonadotrophin was administered. Following the administration of PMS, a peak in the ascorbic acid concentration was reached at midnight — as was the case in the controls.

Table 4 shows the results in animals to which PMS was administered after fasting for 36 hours. The fasting alone caused a decrease in the concentration of ascorbic acid in the blood of 54 per cent on the average. The lowest level was noted a further two hours after the injection of PMS, *i. e.* —60 per cent. The values then once more rose and reached at 12 noon on the following day, *i. e.* 24 hours after the administration of PMS, —39 per cent. — It was thus not possible on the basis of this experiment to demonstrate with certainty that PMS causes changes in the concentration of ascorbic acid in

Rab-bit no.	Nor-mal value	Time:											
		12 ⁰⁰	13 ³⁰	14 ⁰⁰	15 ⁰⁰	16 ⁰⁰	18 ⁰⁰	21 ⁰⁰	24 ⁰⁰	03 ⁰⁰	06 ⁰⁰	09 ⁰⁰	12 ⁰⁰ hrs.
Table 1.													
I	0.92				0%		0	+13	+25	+26	+9	-11	+30%
II	1.20				-15%		+8	0	+19	+14	-23	-7	0%
III	1.05				+33%		+50	+48	+81	+62	-24	-24	0%
IV	1.04				+22%				+44				+13%
XI	1.38				+7%				+67				-9%
Mean figure					+9%		+19%	+20%	+47%	+34%	-13%	-14%	+7%
Table 2.													
X	1.60	-34%				-39	-39	-46	-54	-59	-61		-58%
V	1.18	-41%				-51	-61	-69	-62	-59	-53		-53%
VI	1.46	-58%				-68	-74	-73	-71	-66	-66		-64%
Mean figure		-44%				-53%	-58%	-63%	-62%	-61%	-60%		-58%

Variations in the concentration of ascorbic acid in the blood of normal female rabbits during 24 hours. The figures at 12 noon show mg/100 ml. of whole blood. The changes are expressed as a percentage, in relation to the value at 12 noon. The mean figures are an expression of the percentage variations only.

Table 2.

Blood ascorbic acid concentration after 30 hours' fasting. The normal figure shows the level at 12 noon on the day preceding the beginning of the fast. For the meaning of the figures, see text to Table 1.

Rabbit no.	Normal value	Time:									
		12 ⁰⁰	13 ³⁰	14 ⁰⁰	15 ⁰⁰	16 ⁰⁰	18 ⁰⁰	21 ⁰⁰	24 ⁰⁰	03 ⁰⁰	06 ⁰⁰

Table 3.													
1	0.45	+22%	+56	+100	+111	+56%							
2	1.15	-4%	0	+17	-17	-17%							
3	1.25	+8%	+16	+32	+28	+4%							
4	0.76	-5%	-3	+42	+64	+26%							
5	1.55	-13%	-13	+5	+5	+6%							
6	1.18	-6%	-32	+26	+26	+8%							
7	0.60	0%	+3	+13	+25	+17%							
8	0.65	-17%	-17	-12	+37	+31%							
9	0.90	0%	-1	+39	+89	-22%							
Mean figure	-2%	-5%	+30%	+43%	+10%								

Table 4.

I	0.90	-60%	-64	-64	-57	-49%
II	1.20	-48%	-60	-59	-55	-48%
III	1.05	-54%	-56	-52	-43	-20%
Mean						
figure	-54%	-60%	-58%	-51%	-52%	-39%

Table 3.

Variations in the blood ascorbic acid after intravenous injection of 450 I. U. PMS. For the meaning of the figures, see text to Table 1.

Table 4.

Blood ascorbic acid in rabbits to which 450 I. U. PMS was administered intravenously after 36 hours' fasting. The normal values give the blood ascorbic acid concentration at 12 noon on the day preceding the beginning of the fast. See also text to Table 1.

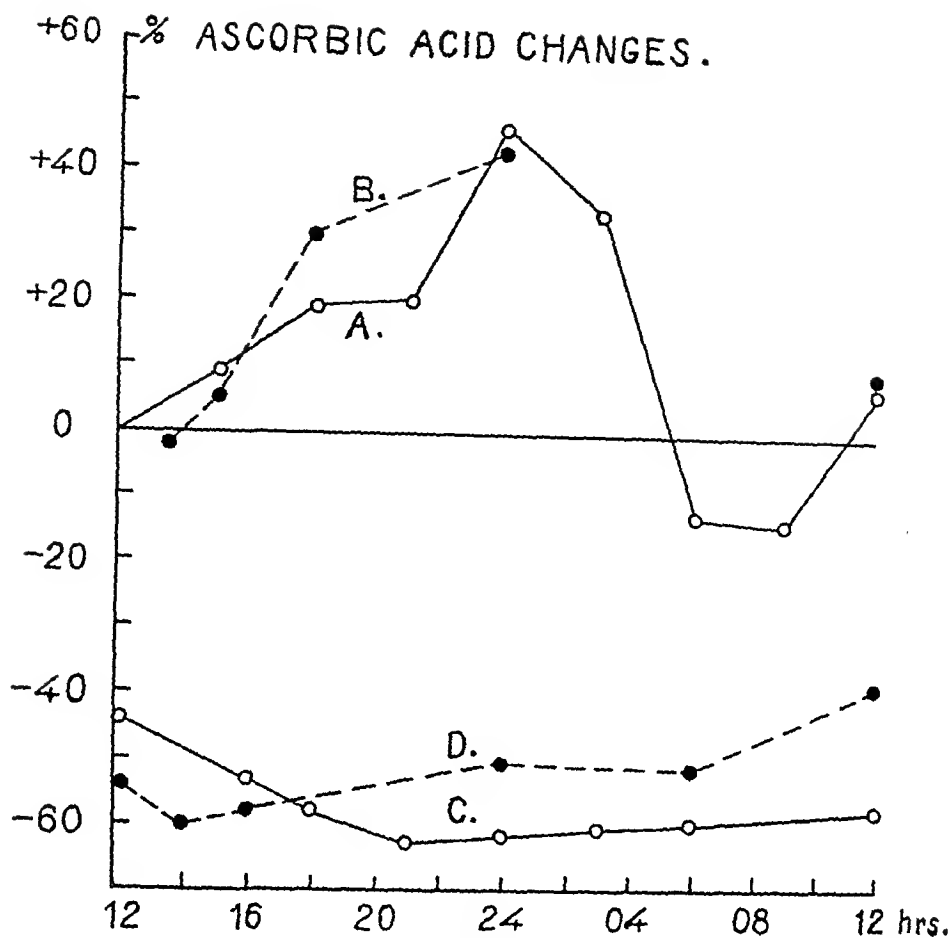


Fig. 1.

A: Normal animals (Table 1).

B: 450 I. U. PMS administered to normal animals (Table 3).

C: After 30 hrs. fasting (Table 2).

D: 450 I. U. PMS after 36 hrs. fasting (Table 4).

latively constant (*v. fig. 1*). It can therefore be assumed that the 24-hour variations in the ascorbic acid level in the blood correspond to its absorption from the intestinal tract.

As can be seen from the results, it was not possible to show that PMS had any definite effect on the ascorbic acid concentration in the blood either in normal or in castrated female rabbits. It is difficult without further study to give the reason why the results reported here differ from those of

Lardy, Casida & Phillips (1944). It is not, however, impossible that the above authors failed to pay sufficient attention to the 24-hour variations which normally occur in the ascorbic acid concentration in the blood.

SUMMARY

Under normal conditions, the ascorbic acid concentration in the blood of normal and castrated female rabbits shows typical 24-hour variations with a maximum at midnight and a minimum in the morning. After 30—36 hours' fasting, on the other hand, the ascorbic acid concentration remains at a relatively constant but low level.

The administration of pregnant mare serum gonadotrophin (450 I. U. Antex Leo intravenously) to normal and spayed rabbits produces no definite changes in the blood ascorbic acid content either in normal or in fasting animals.

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LYMPHOCYTOPENIA AND EOSINOPENIA AFTER PROLONGED INTRAVENOUS ADRENALINE INJECTION ON MAN

BY

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It is generally believed that injections of adrenaline cutaneous as well as intramuscular, induce a transient leucocytosis in the peripheral blood. This increase in leucocytes is characterized by a greater and swifter increase in lymphocytes within one hour as compared with the increase in segmented nuclear granulocytes, which is smaller but of longer duration. In animal experiments an increase in immature granulocyte forms and a changed function of the bone marrow have been recorded, whereas in man a statistically significant increase in rod nuclear granulocytes has been found, and is observed even when the increase in mature forms has ceased (*Hortling*, 1948). The eosinophil leucocyte count is said to decrease a few hours after the administration of adrenaline (cit. *Halberg*, 1949). In the investigation by *Hortling* (1948) the decrease was, however, insignificant. Lymphopenia after adrenaline injection has been recorded in animal experiments by a few investigators. Subcutaneous (*Harlow & Selye*, 1937, *Latt & Henderson*, 1937) and repeated intravenous injections (*Malmeljac et al.*, 1948) as well as intraperitoneal injections (*Gellhorn & Frank*, 1948) were tried. In the two latter investigations, which were published when the present work was going on, the lym-

phopenia could not be established in adrenalectomized animals. As there is evidence that administration of adrenaline increases the concentration of adrenocortical hormone in the adrenal vein (*Vogt*, 1944), the last mentioned observations are of interest in connection with the fact that lymphopenia and eosinopenia have been recorded both in animals (*White & Dougherty*, 1945) and in man (*Hills et al.*, 1948) after the administration of corticotrophic and certain cortical hormones. On the other hand it has not been proved that the administration of adrenaline in man will induce lymphocytopenia, which is the more remarkable, as the effect of adrenaline on the animal organism appears to differ in some respects from that observed in man (compare *Hortling*, 1948). It therefore seemed to be of interest to investigate the effect of prolonged adrenaline administration on the peripheral blood picture in man.

METHODS AND MATERIAL

In the experiments an infusion apparatus, constructed by *Lahti* and *Östling* according to the same main principles as applied by *Kurode* and *Straub*, was used. The apparatus allows of an even, adjustable infusion rate for periods of several hours. The needle is inserted into a cubital vein. Out of consideration for the patient the infusion period did not exceed one hour. The preparation Exadrin Astra was used.

The doses varied between 0.2 and 0.3 γ /kg./body weight per minute. According to the findings of various investigators summarized by *Pekkarinen* (1948) both adrenals at rest secrete about 0.08 γ /kg./min. and with increased secretion the average output is 0.2—1.0 γ /kg./min. The dose administered in the present study is thus physiologically possible.

All the subjects tolerated the infusion well. Symptoms such as palpitation, trembling and agitation were present in varying degrees in all of them during the course of the adrenaline administration. The symptoms disappeared within a few minutes after cessation of the infusion. Flushing of the face was frequently observed for a short time after the adrenaline admini-

stration has been suspended. The period of infusion varied between 40 and 60 minutes.

The peripheral blood picture was determined by tests from the finger tip of fasting persons. The first drops were not used. The erythrocyte, reticulocyte and leucocyte counts as well as the hemoglobin percentage according to Sahli were determined. In 6 experiments the composition of the arterial blood was also determined in the radial artery. The blood tests were taken before the beginning of the adrenaline infusion, 10 minutes later, approximately in the middle of the infusion, and immediately before the administration was discontinued. Further tests were made $\frac{1}{2}$, $1\frac{1}{2}$ and $2\frac{1}{2}$ hours after cessation of the adrenaline infusion. The erythrocyte count was determined by means of Leitz counting chamber, and 200 small squares were counted. For the determination of the leucocyte count the same instrument was used and 12 large squares were counted. A differential count was made on 400 leucocytes in smears stained according to May-Grünwald-Giemsa. The reticulocyte count was determined in per cent of 1000 erythrocytes. The hemoglobin percentage was determined with the aid of Hellige's normal hemometer. The averages were calculated on the checked values.

The experiments were carried out on 13 hospital patients, 4 of whom had some infection; the others had no infections and no blood, nervous or other severe disorder. The control cases were in the main persons in good health.

RESULTS

The results of the 13 tests are presented in Table 1 and Figs. 1 and 2. Fig. 1 shows the variations in the averages for the absolute cell counts within the different cell types. The differences in per cent of the values before the beginning of the tests, are presented in Fig. 2. The variations in the different cell types to which the present study applies are described in details as follows: The *segmented nuclear granulocyte* count increased from an average of $3792/\text{mm}^3$ to a maximum of $5367/\text{mm}^3$ (the increase was 42 per cent) at the end of the

Table 4.

Variations in the percentage of rod nuclear granulocytes and lymphocytes in the peripheral blood in the 13 adrenal line tests.

	1.		2.		3.		4.		5.		6.		7.		8.	
	rod	ly	rod	ly	rod	ly	rod	ly	rod	ly	rod	ly	rod	ly	rod	ly
Before	1.75	34		23.5	5.25	26.25	8	28	3	33	4	32.75	6.5	20	6.5	39.5
During adrenaline infusion	2.25	44.25	2	27.5	2.75	36.5	7	30.5	1.75	51.75	2.5	47.25	5.75	22.5	2.75	55.75
	3	51.25	3	32	4	35.5	5.5	42.25	2.75	48.5	1.75	45	4	31	6.75	46
1/2 hour after	3.5	25	5	30.5	3.75	38	8.5	36.5	2	49	3.25	48.25	8.25	33.75	7.75	44
1 1/2 »	7	22.25	6.25	12	9.25	6.75	12.25	8.5	5.5	20.25	6.5	25	18.25	9.5	13.75	27.75
2 1/2 »	7.5	15.5	6.75	8.25	14.5	10	14.75	12	6.5	21.5	9.5	18.5			20.25	23.5
8 »			7.25	13.25	7.5	11	8	15.75								

adrenaline infusion. After suspension of the infusion it decreased slightly and then again slowly increased to a maximum of $6291/\text{mm}^3$ $2\frac{1}{2}$ hours later (67 per cent). The rod nuclear granulocyte count (metamyelocytes and myelocytes included) increased during the adrenaline infusion from $352/\text{mm}^3$ to $623/\text{mm}^3$ (77 per cent) at the end of the infusion; a maximum of $1079/\text{mm}^3$ (207 per cent) was reached $1\frac{1}{2}$ hour after discontinuation of the infusion. During and after the administration of adrenaline a slight increase in *metamyelocytes* in the peripheral blood was recorded in 9 cases and 2 of them also showed a slight addition of *myelocytes*. The *lymphocyte* count increased from $1740/\text{mm}^3$ before infusion, to a maximum of $4820/\text{mm}^3$ (177 per cent) as a consequence of the adrenaline effect. Following suspension of the infusion a rapid depression occurred, so that a level of $986/\text{mm}^3$ (—43 per cent) was noted $\frac{1}{2}$ hour later. This depression could still be recorded $2\frac{1}{2}$ hours after infusion had been suspended (—20 per cent). The *eosinophil granulocytes* behaved in the same way as the lymphocytes. From the initial level $158/\text{mm}^3$ the average rose during the adrenaline administration to a maximum of $269/\text{mm}^3$ (70 per cent) and declined to $65/\text{mm}^3$ (—59 per cent) $1\frac{1}{2}$ hours after suspension of the infusion. The *monocytes* also showed an increase during the adrenaline infusion from $507/\text{mm}^3$ to a maximum of $1016/\text{mm}^3$ (100 per cent) and a decrease half an hour after cessation of the infusion to $356/\text{mm}^3$ (—30 per cent), when the level approached normal values.

The regular appearance of the variations was remarkable. Common to all the tests were the increase in rod nuclear granulocytes and the lowering of the lymphocyte count below the initial level on suspension of the adrenaline infusion, while an increase in lymphocytes during the adrenaline administration was recorded in 12 tests out of 13. The variations in the eosinophil granulocyte count described above, as well as the decrease of monocytes below the initial values were also found in 12 of the 13 tests. Statistical treatment was therefore considered unnecessary. — In Table 1 the results

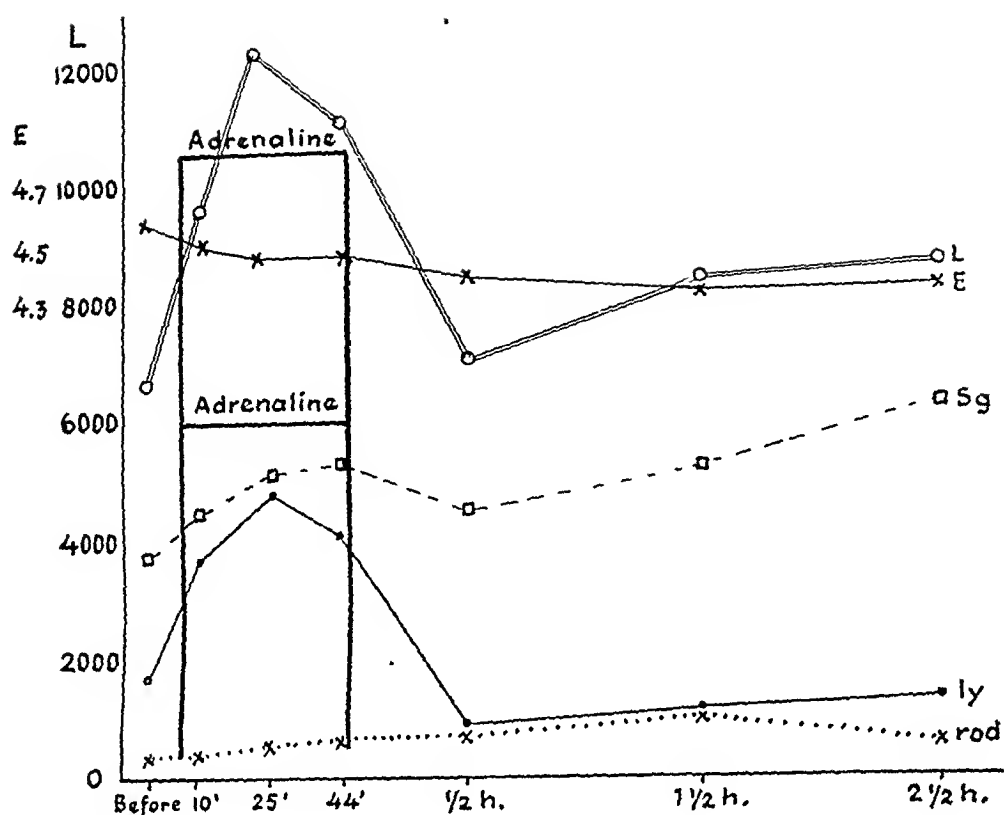


Fig. 1.

Variations in absolute count of erythrocytes (E), leucocytes (total count, L), segmented nuclear granulocytes (sg), lymphocytes (ly) and rod nuclear granulocytes (rod) during and after intravenous adrenaline infusion. Mean values of 13 tests.

of the differential count for rod nuclear granulocytes and lymphocytes in per cent are shown. In some cases at least, the variations are considerable.

In order to eliminate differences in the distribution of the leucocytes in the peripheral blood as a possible cause of the variations found, some control experiments were carried out. In 6 tests, injection of distilled water in the same quantity and with the same technique as used in the adrenaline tests did not affect the peripheral blood picture to any considerable degree. In 6 adrenaline tests, blood was also taken from the radial artery simultaneously with some tests from the finger tip, and the same variations were recorded in the arterial blood

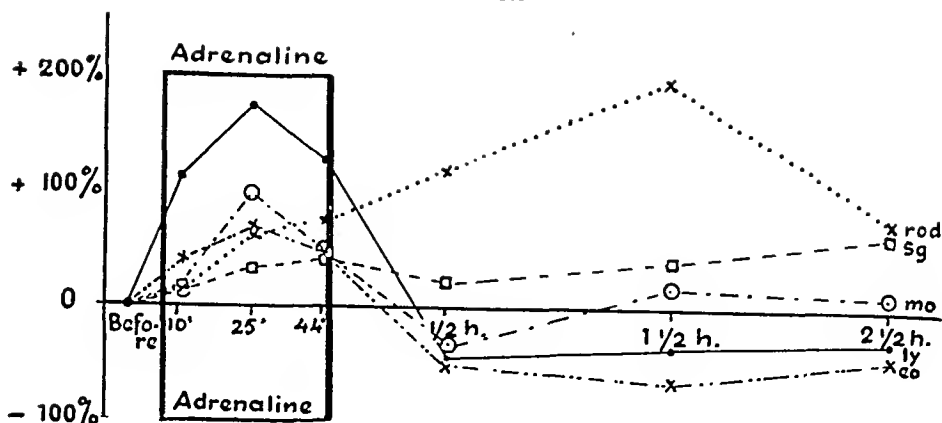


Fig. 2.

Variations in per cent of rod nuclear granulocytes (rod), segmented nuclear granulocytes (sg), monocytes (mo), lymphocytes (ly) and eosinophil granulocytes (eo) during and after intravenous adrenaline infusion. Mean values of 13 tests.

as in the peripheral blood. The values in the arterial blood were in the main somewhat lower than the values found in the blood from the finger tip. Previous experiments by *Hortling* have proved that the solvent of adrenaline in the preparation Exadrin Astra, which was used in the present study, does not affect the peripheral blood picture on intramuscular injection and that the variations in the leucocyte picture after adrenaline are also present in venous blood.

In Fig. 1 the mean values for *erythrocytes* during infusion are also shown. A slight tendency to fall is seen. This also applies to *hemoglobin*. The average for erythrocytes fell from 4.581 millions/mm³ before to a minimum of 4.338/mm³ 2 1/2 hours after the adrenaline infusion, while the hemoglobin percentage fell from an average of 92 to the lowest value 88, 1/2 hour after suspension of the adrenaline infusion. Colour index averaged 1.00 before and 1.05 1/2 hour after infusion. The small variations, which were not regular, do not seem to suggest that appreciable variations in the blood concentration occurred. The *reticulocytes* were examined at 9 tests. No apparent alterations were found.

DISCUSSION

The decrease in granulocytes appearing as a late effect during and after prolonged adrenaline infusion in doses not exceeding the amount which could be physiologically present in the adrenal veins, has also been recorded after intramuscular and subcutaneous injection, though not regularly. A new observation in man is the appearance of a sudden decrease of lymphocytes count after the cessation of the adrenaline infusion to values lower than the initial ones. The lymphocyte values increased during the adrenaline administration. The eosinophil granulocytes behave in the same manner and so do also the monocytes, though more transiently. Hence 1½ hours after cessation of adrenaline administration the leucocyte picture in the peripheral blood very much resembles that seen after administration in man of adrenocorticotrophic hormone and of 17-hydroxycorticosterone (*Hills et al.*, 1948). These observations suggest the possibility that the administration of adrenaline stimulates the adrenal cortex to increased activity, though whether this effect is a direct one or mediated through the pituitary gland cannot at present be stated. Thus there appears to be evidence that continuous intravenous adrenaline administration induces a reasonably constant change in the leucocyte count in the peripheral blood, a change which cannot be attributed to alterations in the distribution of these cells in the blood or to altered blood concentration. The most probable cause of this change appears to be a change in the regulation of the cell counts concerned. The sudden fall in the number of certain cells might possibly be attributed to a disintegration of these cells (compare *White & Dougherty*, 1945).

Experiments aiming at a closer examination of the nature of the effect of prolonged intravenous adrenaline administration on the blood picture, as well as on the blood chemistry are in progress.

SUMMARY

½—2½ hours after intravenous adrenaline infusion in man for a period of about 50 minutes, an increase in the ab-

soluble rod nuclear and segmented nuclear granulocyte counts in the peripheral blood occurred, while the lymphocyte and eosinophil granulocyte counts decreased during the same period to values below the initial ones. In the course of the adrenaline infusion all these cell types increased. The observations are based on 13 tests and the variations found were constant. It is possible that the variations in the blood picture after cessation of adrenaline infusion may be attributed to increased activity of the adrenal cortex.

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THE SIGNIFICANCE OF RENAL FUNCTION FOR THE EFFECT OF DESOXYCORTICOSTERONE ACETATE (DCA) IN ADDISON'S DISEASE*)

BY

ROLF LUFT and BJÖRN SJÖGREN

Disturbed water and salt metabolism is a cardinal symptom in Addison's disease. It causes dehydration of the patient, as well as hypotension and, to some extent, asthenia.

The disturbed fluid balance is manifested by an incapacity to respond in the normal way to rapid changes in the fluid supply. When a fairly large quantity of fluid is administered, e. g., in a water test or with Kepler's test, this is excreted more slowly than normal and the urine does not undergo maximal dilution (*Robinson et al.*, 1941, *Levy et al.*, 1946).

Talbott et al. (1942), in cases of adrenal cortical insufficiency, found a reduced glomerular filtration rate and renal plasma flow, the filtration being proportionately more reduced. These authors also discuss the earlier literature on this subject. Recently *Waterhouse & Keutmann* (1948) demonstrated a marked reduction of glomerular filtration and renal plasma flow in their cases of Addison's disease.

In important animal experiments a number of authors have confirmed the significance of the adrenal cortex for re-

*) The DCA used in the present investigations was »Percorten» Ciba which was kindly placed at our disposal by Ciba Produkter AB, Stockholm.

nal function (*Porak & Chabanier*, 1914, *Marshall & Davis*, 1916, *Hartman et al.*, 1927, *Swingle*, 1927, *Harrop et al.*, 1933, *Silvette & Britton*, 1933, *Harrison & Darrow*, 1939, *Gersh & Grollman*, 1939, *Kottke et al.*, 1942, *Gaunt*, 1946). The most consistent findings after adrenalectomy were an increased nonprotein nitrogen and urea concentration in the serum, delayed water excretion and reduced glomerular filtration.

Patho-anatomical investigations have demonstrated the occurrence of tubular degeneration in cases of Addison's disease (*Barker*, 1929). These changes have also been observed in animals after adrenalectomy (*Swingle*, 1927, *Hartman et al.*, 1927), and may be prevented by the administration of cortical extract (*Simpson & Korenchevsky*, 1935). However, other investigators have not been able to confirm these findings (*Harrop & Weinstein*, 1932, *Gersh & Grollman*, 1939).

Some French investigators (*de Gennes et al.*, 1947) have pointed out that the occurrence of renal damage should be taken into consideration in the treatment of Addison's disease since this might play a part in the patient's reaction to DCA. »L'hypertension sèche« might be produced in these patients by DCA.

The secondary effects seen in cases of Addison's disease in the course of treatment with DCA are, principally, peripheral edema, circulatory insufficiency and hypertension (*Ferrebee et al.*, 1939, *Mc Cullagh & Ryan*, 1940, *Thorn et al.*, 1942).

In connection with an investigation on the effect of DCA under varying conditions, the present authors observed that the administration of DCA to patients suffering from renal damage could give rise to edema and hypertension. In healthy persons the fluid retention and increase of blood pressure with the same kind of treatment were only moderate (*Luft & Sjögren*, 1949). In this paper, the factors necessary for such secondary effects in cases of Addison's disease are studied, with particular reference to their connection with disturbances of renal function.

CLINICAL INVESTIGATIONS

The investigations comprise five males and one female, all with definite clinical signs of Addison's disease. The data are given in Table 1 in which the results of Kepler's test are also reproduced.

*Table 4.**Clinical data in six cases of adrenal insufficiency.*

Patient	Sex	Age at Onset	Duration of Disease	Blood Pressure Mm. Hg.	Kepler's Test. Index
1. 2/48 O. L.	M	35 years	6 months	90/55	12
2. 630/48 S. A. J.	M	23 years	9 months	100/65	7.8
3. 684/48 N. A. S. N.	M	21 years	9 months	90/50	9
4. 596/48 K. I. E.	M	53 years	7 months	80—120/70	—
5. 476/48 K. E. L.	M	27 years	1—2 years	115/70	19
6. 90/48 E. H. M. L.	F	44 years	5 years	120/80	12

The effect of large doses of DCA and sodium chloride was tested in all the cases. The results are presented in Table 2.

It can be seen from this table that three patients reacted with a moderate rise of blood pressure. One of these patients reached a blood pressure of 150/100 mm. Hg. but only after one month of treatment with large doses of DCA and saline. Simultaneously, the hematocrit and red blood corpuscle values

Table 2.
Effect of large doses of DCA and sodium chloride in adrenal insufficiency.

Patient	Daily Dose		Duration of Administration	Maximum Weight Increase kg.	Edema	Blood Pressure Mm./Hg		Hematocrit		Red Blood Cells Mill./Mm ³	
	NaCl gm.	DCA mg.				Before	After	Before	After	Before	After
1.	5	10-15	38 days	3.5	—	90/60	140/80	38	30	3.6	2.9
2.	20	20	47 days	6.2 (3.0)	+	85/55	150/95	58	35.5	5.1	3.5
3.	10	20	22 days	8.0 (4.0)	+	90/55	130/80	56	34	4.9	3.0
4.	5	20	8 days	4.2	+++	110/75	110/75	36	29	3.6	2.8
5.	—	—	—	—	—	—	—	—	—	—	—
6.	5	5	40 days	0	+	120/80	175/100	—	—	—	—

fell, indicating increased plasma volume. The increase in weight was considerable but the fact that the patient was dehydrated by being put on a diet deficient in salt for a week before the DCA test should be taken into account. The real increase in weight was actually only 3—4 kg. Only slight edema was found, and this disappeared in spite of continued administration of DCA.

Case 4 reacted with rapidly developing edema which made it necessary to discontinue treatment with DCA. However, the blood pressure remained unchanged. The patient showed the usual signs of increased plasma volume.

In Case 6 a rapid and marked increase in the blood pressure set in in spite of the decrease of DCA to a quarter of the original dose.

These three different types of reaction to DCA are illustrated in Figs. 1—3.

The renal function was also examined in the same cases of Addison's disease (Table 3).

Table 3.
Renal function in adrenal insufficiency.

Patient	Urine		NPN mg. pr. cent	Water Test (1000 ml.)			Clearance Tests	
	Album- inuria	Casts and Red Cells		Excretion in 4 Hours ml.	Maximum Conc. Spec. Grav.	Maximum Dilut. Spec. Grav.	Glomeru- lar Filtra- tion ml./min.	Renal Plasma Flow ml./min.
1.	—	—	40					
2.	—	—	40	905	1.021	1.011	81	424
3.	—	—	46	<400			81	438
4.	—	+	46—60	825	1.016	1.008	18	176
5.	—	—	57	465	1.022	1.007		
6.	—	—	40—60	855	1.012	1.005	44	

S. Å. J. 8 24 YEARS
ADRENAL
INSUFFICIENCY

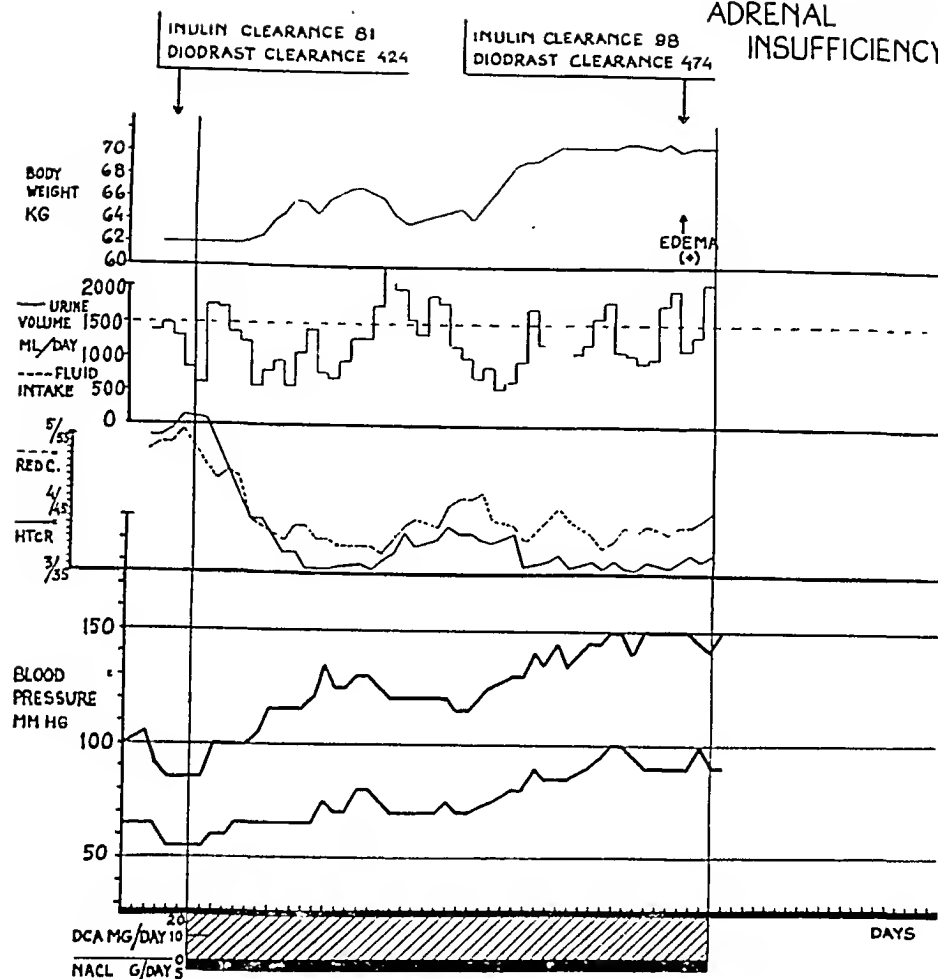


Fig. 4.

Case 2. Adrenal insufficiency in a 24 years old male. Effect of DCA and sodium chloride on blood pressure, red blood cell counts and hematocrit readings, fluid balance, body weight and clearance values.

It emerges clearly from the table that changes in renal function were noticed in all the cases examined. The water test showed, above all, inadequate capacity to dilute and to concentrate. However, it should be emphasized that the water test can be analysed only when the fluid balance of the patient is known. Thus, Case 3 showed a comparatively normal

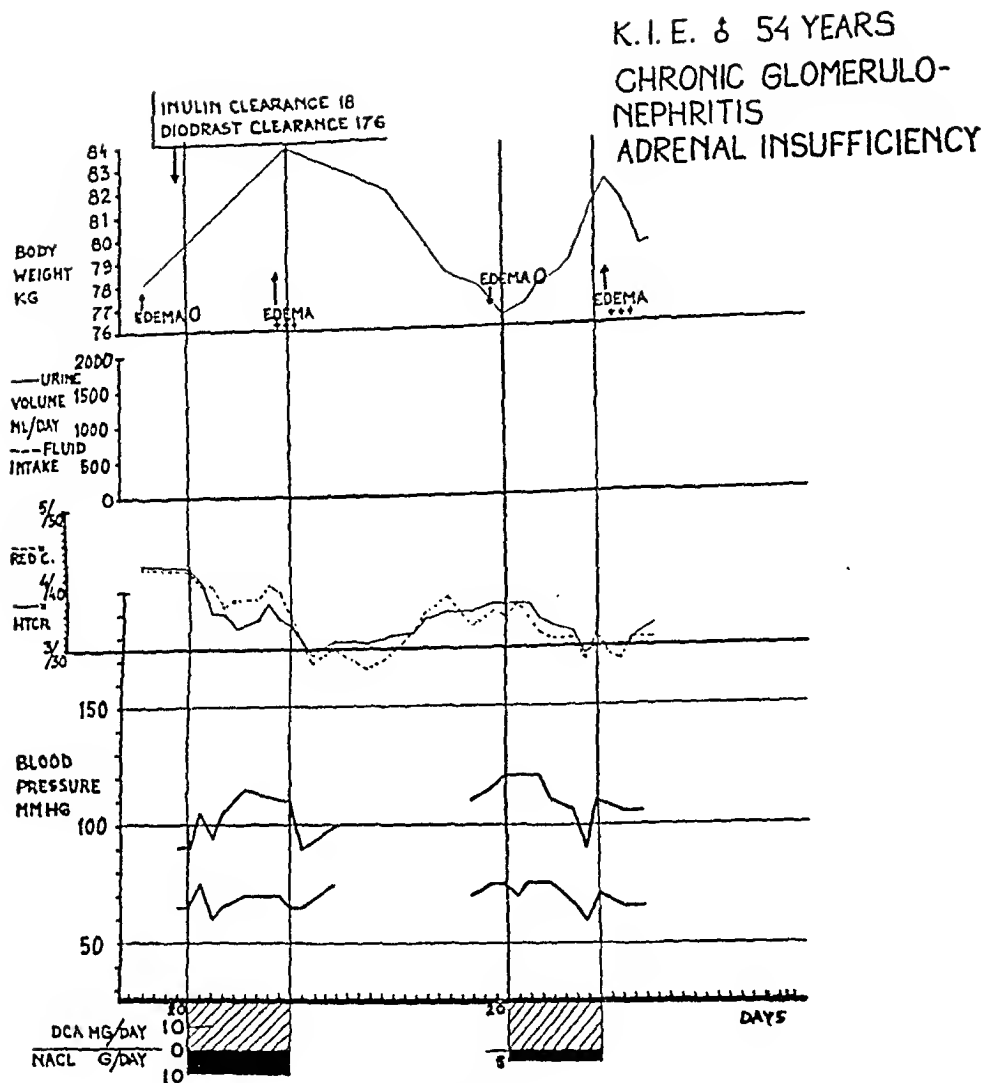


Fig. 2.

Case 4. Adrenal insufficiency and chronic glomerulo-nephritis in a 54 years old male. For legend, see Fig. 1.

water test at the first examination when additional sodium chloride had recently been given. When the test was repeated a few days later, the patient revealed the typical signs of retarded excretion. This is in contrast to the findings of *Reforzo-Membrives et al.* (1945), who deny the influence of additional saline, when given previous to the water test.

In all these cases examinations of the glomerular filtration and renal plasma flow show lowered values. In the first cases,

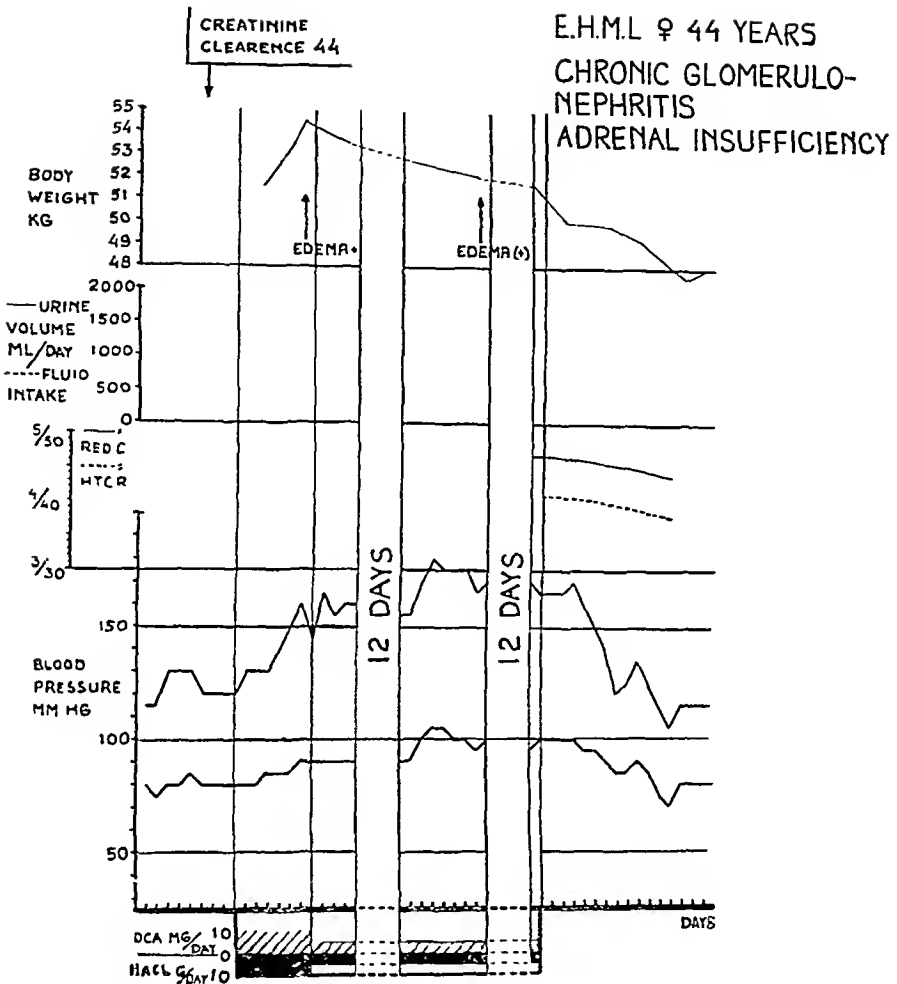


Fig. 3.

Case 6. Adrenal insufficiency and chronic glomerulo-nephritis in a 44 years old female. For legend, see Fig. 1.

the renal function was only moderately reduced, and in the two lastmentioned cases the clearance values were considerably decreased.

Thus the cases in which secondary effects in the form of edema or rapidly developing hypertension developed on administration of DCA also showed the least satisfactory renal function.

In three cases the authors were able to study the renal function after a period of DCA administration (Table 4).

Table 4.

Effect of DCA on renal function in three cases of Addison's disease.

Patient		Clearance Tests (Inulin-Diodrast)			Kepler's Test
		Glom. Filtr. ml./min.	Renal Plasma Flow ml./min.	Filtration Fraction	
1.	Before DCA				
	After »	110			
2.	Before DCA	81	424	0.19	7
	After »	98	474	0.21	255
3.	Before DCA	81	438	0.18	
	After »	130	442	0.29	

From the above table it is evident that DCA was capable of improving the filtration rate in two of the cases. In one of these, the water test was repeated and found to be normal. In Case 1 the inulin clearance was studied only during the course of Percorten treatment and showed a normal value.

Chronic DCA administration, on the other hand, failed to improve renal function in Cases 4 and 6, the water test still disclosing hyposthenuria whilst the nonprotein nitrogen value remained well above the normal. In Case 4, death occurred owing to uremia and autopsy revealed a chronic glomerulonephritis.

To sum up, the following observations was made:

1. Disturbed renal function occurred in all the cases of Addison's disease examined.
2. Routine examinations and water tests did not allow of differentiation between cases with functional and organic renal damage.
3. Only by renal clearance tests was it possible to detect the presence of organic renal damage with any degree of probability.

4. The administration of DCA and sodium chloride improved the disordered renal function in the cases without organic renal disease.
5. The administration of DCA caused edema or hypertension only in cases with organic renal disease.

DISCUSSION

The disturbances of renal function observed in the present cases of Addison's disease were of two different kinds, viz., those with only moderate changes in the glomerular filtration and renal plasma flow, and those with markedly reduced clearance values. The former were improved by the administration of DCA and sodium chloride. We presume that these cases had a functional renal disturbance, while the latter cases showed manifestations of a complicating organic renal disease. In one of the cases the organic renal damage, i. e. a chronic glomerulonephritis, was verified at autopsy.

It may be assumed that there is a connection between the decreased clearance values and an altered vascular tone in the cases with moderate changes in the clearance values. The lowered vascular tone is manifested by hypotension and a low filtration fraction.

However, *Waterhouse & Keutmann* (1948) on the basis of their clinical observations state that such a relation between hypotension and lowered filtration is hardly likely. We feel, however, that these authors have paid too little attention to the possibility that the very low clearance values in some of their cases might be due to complicating organic renal disease such as chronic glomerulo-nephritis. The same authors did not find any improvement of renal function during DCA treatment. In contrast to *Waterhouse & Keutmann* and in agreement with the present authors, *Talbott et al.* (1942) were able to increase glomerular filtration with DCA in five cases of Addison's disease.

As another example of the relation between vascular tone and renal function, we have reproduced in Table 5 the effect of Gynergen in two cases of postural hypotension. In these

cases Gynergen increases the blood pressure and at the same time brings the renal function back to normal.

Table 5.

Renal function in two cases of postural hypotension.

Patient		Blood Pressure in Recumbant Posture mm. Hg.	Water Test (1000 ml.) Excretion in 4 Hours	Clearance Tests Glomerular Filtration
1. K. O. V. E. 3/47	Before Gynergen	90/60	115	29 (creatinine)
	After Gynergen	175/125	700	90 (inulin)
2. E. G. J. 1078/46	Before Gynergen	90/60	340	32
	After Gynergen	170/120	1100	132

} creatinine

Our investigations, therefore, suggest that there is a relation between a decreased vascular tone and the lowered glomerular filtration seen in uncomplicated cases of Addison's disease. However, we do not as yet know the significance of the duration of the disease with regard to a decrease in the clearance values. In our own three cases, where an improvement of the filtration rate was obtained by the administration of DCA, the duration of the disease was only 6—12 months.

It has been demonstrated by animal experiments that DCA in large doses can produce organic renal damage (*Selye et al.*, 1943—44). A few investigators have been unable to verify these findings (*Bechgaard & Bergstrand*, 1949). *Knowlton et al.* (1946), on the other hand, found that renal damage due to nephrotoxic serum was aggravated by the administration of DCA. The question as to whether a relatively long period of DCA administration may produce renal damage in patients

suffering from Addison's disease is, therefore, an important one, but has not yet been elucidated. We were able to treat a patient suffering from postural hypotension with 20 mg. of DCA and 10 gm. of sodium chloride daily for a total period of six months (*Luft, Santesson & Sjögren, 1948, Luft & Sjögren, 1949*), but this treatment produced no further decrease in the originally low clearance values.

In the present investigations, the diagnosis of organic renal damage in two of our cases proved of great value for both the therapy and prognosis. The fact that both these cases reacted differently was of particular interest. One developed edema without any rise in the blood pressure, the other had a rapid rise of blood pressure but no edema. The same type of reaction to DCA was observed by us in cases of organic renal damage without adrenal insufficiency. We hope to return to this question.

The two cases with organic renal damage could only be treated with small doses of DCA, insufficient to affect the patient's asthenia to any appreciable extent. In these cases testosterone propionate was of special therapeutic value since it improved the patient's condition considerably (*Luft & Sjögren, 1949*).

It is thus possible that the immediate complications due to DCA treatment in Addisonian patients may often be caused by a complicating renal disease.

SUMMARY

The renal function was studied in six cases of Addison's disease. Disturbed renal function was ascertained in all six, viz. increased non protein nitrogen values, abnormal water tests and reduced glomerular filtration and renal plasma flow. In two cases the clearance values were moderately lowered, in two others a more marked reduction of the renal function was observed. In two of the cases — both with only moderately reduced renal function — DCA and sodium chloride caused improvement of the renal function. In the authors' opinion, the disturbed renal function prior to treatment in these two

cases was due to functional damage, largely connected with an altered vascular tone.

In the two cases with a more severe reduction in the renal function, a complicating organic renal disease probably occurred. This was verified at autopsy in one of the cases. The administration of only small doses of DCA in both these cases produced complications in the form of edema or hypertension.

The authors consider that a disturbed renal function is of considerable significance in the immediate occurrence of complications arising from the usual treatment of Addison's disease with DCA and sodium chloride.

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THE FUNCTION OF THE ENDOCRINE GLANDS IN DIABETES MELLITUS

A CLINICAL STUDY OF 107 CASES

BY

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For a number of years following the discovery of insulin it has been commonly accepted that diabetes mellitus in man is due to a deficient pancreatic secretion of insulin. A new and important fact in the problem of the etiology of the disease appeared when *Houssay* (1930, 1931), and *Houssay & Biasotti* (1930) showed that hypophysectomy in pancreatectomized animals greatly alleviated the diabetes, thus suggesting that the pituitary gland secretes a diabetogenic factor. Later (1937) *Young* was able to produce a permanent diabetic state in dogs by injections of anterior pituitary lobe extracts. Further, it was demonstrated that this kind of diabetes is most readily produced in adult dogs, in which the onset is preceded by an increase in weight, while injections of pituitary extracts in puppies cause acceleration of growth, diabetes occurring only with intense treatment; in the latter case growth ceases (*Young*, 1941 b, 1944, 1945).

These experiments led to the investigation of the significance of other endocrine glands in the pathogenesis of the disease. The results of animal experiments were reviewed by *Houssay* (1937). The most outstanding facts, in addition to

the importance of the pituitary gland, are as follows: Thyroid extract may cause diabetes in partly pancreatectomized dogs but not in normal dogs. Sometimes the diabetes continues after stopping thyroid feeding (metathyroid diabetes, *Houssay*, 1944). Thyroidectomy increases the sensitivity to insulin, but does not cure diabetes. Adrenalectomy produces hypersensitivity to insulin, and this is also seen if the medulla only is removed. Pancreatic diabetes is alleviated by adrenalectomy. *Ingle* (1941, a) showed that administration of 17-hydroxy-11-dehydrocorticosterone to normal rats caused hyperglycemia and glycosuria (adrenal steroid diabetes). *Conn et al.* (1948) were able to produce a temporary diabetes mellitus in man by injections of purified pituitary adrenocorticotrophic hormone. They conclude that »so far as pure pituitary fractions are concerned, adrenocorticotrophic hormone has been found to have the greatest diabetogenic effect of any tested to date.«

Evidence of diabetes mellitus in man caused by endocrine disturbances other than pancreatic deficiency is scanty. Acromegaly resulting from an eosinophilic adenoma of the pituitary gland is fairly often associated with diabetes (yet, according to *Wilder* (1940), only in 6 to 9 per cent of all cases). Also, pituitary basophilism is fairly frequently combined with diabetes. Alleviation of diabetes by destruction or removal of the pituitary gland (the *Houssay* phenomenon) has been reported four times in man (*Lyall & Innes* (1935), *Chabanier et al.* (1936), *Kotte & Vonderahe* (1940), *Feldman et al.* (1947)).

Tumours (adenomas) of the adrenal cortex are sometimes complicated by diabetes. *Russi et al.* (1945) found that diabetes occurred five times as frequently in subjects with cortical adenomas as in a general group of 9000 autopsies. *Sprague et al.* (1943) report a case of tumour of the adrenal cortex in which the complicating diabetes was cured following extirpation of the tumour. Diabetes is sometimes seen in patients with a pheochromocytoma. *Duncan et al.* (1944), *Green* (1947) and *Goldner* (1947) report such cases in which the diabetes disappeared following removal of the tumour.

Sprague et al. (1948) describe a case of assumed »steroid

diabetes», associated with Cushing's syndrome, in a 14-year-old boy.

On the other hand, diabetes and Addison's disease may be found in the same patient. According to *Feldman et al.*, in 1947, 16 cases showing this combination were on record. Another case is reported by *Ernberg* (1947), and, recently, *Knowlton & Kritzler* (1949) found reports of 22 instances of this combination in the literature. Either of these diseases may be the first to appear.

It is generally agreed that the incidence of diabetes is greater among patients with hyperthyroidism than in the population as a whole. That hyperthyroidism greatly aggravates a preexisting diabetes is a well known fact. On the other hand, *Weinstein* (1932) believes that diabetes is particularly rare in myxoedema, but this is denied by *Shepardson & Wever* (1934). *Wilder et al.* (1934) report a case in which removal of a morphologically normal thyroid gland markedly increased the glucose tolerance of a patient with severe diabetes. The increase in tolerance roughly paralleled the decrease in the metabolic rate. Similar observations have been reported by *Thune Andersen* (1933).

Only inconclusive evidence exists regarding the significance of the parathyroid glands in human diabetes (*Olmer & Paillas*, 1936, and *Zunz & La Barre*, 1933). The problem of the function of the gonads in human diabetes will be considered later.

There is very little histological work on the endocrine glands in diabetes. In 1914, *Fry* described in pituitary glands from diabetics 1) an increase in the chromophile cells; 2) conversion of chromophile granular cells into colloid or granular masses; 3) areas of degeneration which may be of such size that few of the cellular elements remain in the anterior lobe. Examining the endocrine glands of diabetics, *Krans* (1923) found the pituitary gland to be smaller than normal, with a reduction in the number and size of the acidophile cells, while the chromophobe cells were present in increased number. The adrenal glands were smaller than normal in young subjects.

but, on the contrary, larger than normal in older people. The thyroids showed various changes, but not in any definite direction, while the parathyroids were remarkably small. Regarding the gonads, a few men showed decreased spermatogenesis, and, in women, the ovaries, in some cases, presented deficient follicular ripening.

OWN INVESTIGATIONS

The scope of the present work was to investigate whether non-selected clinical cases of diabetes, submitted to a special examination showed any signs of abnormal functions of the endocrine organs apart from the pancreas. Of course marked endocrine disturbances were not to be expected, but it appeared possible that the clinical and laboratory findings might support, or be inconsistent with the endocrine etiologies suggested by animal experiments and by the occasional cases seen in human pathology.

In the period from May 1947 to August 1948 107 diabetic in-patients in the Medical Department of the City and County Hospital in Odense were examined. This hospital is the centre of medical treatment in the northern part of the island of Fyn in central Denmark. The district served, covers an area of 1144 square km, with a population of 168000, 100000 of these living in the City of Odense, and the remainder partly in smaller towns, partly in the country. Practically all inhabitants are of Danish origin, foreign immigration being insignificant.

The patients included in the investigation were questioned with special reference to signs of endocrine disorders in the history. A clinical examination was performed and the height, weight, blood pressure, blood sugar, basal metabolic rate, total serum cholesterol and serum calcium were determined. Urine analyses were made, the sella turcica X-rayed, the eyes were examined and in more than half the cases the urinary hormonal excretion was estimated.

Statistical evidence.

One woman had a basophile adenoma of the pituitary

gland. Because of the probable special etiology in this case it is omitted in the final analysis. Of the remaining 106 diabetics, 45 were males, 61 females (42 and 58 per cent respectively). 31 males and 34 females received insulin treatment.

As the sex distribution, married or unmarried state, age at the beginning of the disease, and height and weight measurements are considered important in the subject under discussion, these results were obtained from all diabetics treated in the department in 1945 and 1946 and have been added to the data, thus bringing the number of diabetics investigated up to 241. This allowed of a more reliable statistical analysis.

Of the total number of 241, 102 were males, 139 females (42 and 58 per cent respectively), exactly the same proportion as in the cases specially examined.

Table 1.

241 cases of diabetes mellitus, grouped according to age at the beginning of the disease.

Age	Diabetics		Total population		Diabetics per 1000	
	♂	♀	♂	♀	♂	♀
0—9	15	18	13429	13042	1.1	1.4
10—19	15	14	14024	13317	1.1	1.1
20—29	13	9	14845	14794	0.9	0.6
30—39	19	9	13794	13626	1.4	0.7
40—49	12	16	10384	10971	1.2	1.4
50—59	17	33	8294	8249	2.0	3.9
60—69	7	33	5576	5684	1.3	5.6
>70	4	7	3656	4294	1.1	1.6
	102	139	84002	84007		

Age at onset of the disease is seen in Table 1. Evidently these figures must be seen in relation to the size of the various age groups in the general population. This is done by calculating the numbers of hospitalized diabetics per 1000 inhabitants in each age decade. This, of course, does not represent the annual morbidity rate of diabetes in the population, (as these are only the hospitalized diabetics, over a three year pe-

creted as 17-ketosteroids only to a very slight extent (2—3 per cent).

6) An inhibition of the hypophyseal corticotrophin and gonadotrophin secretions, as reflected in subnormal 17-KS values after cessation of testosterone administration, was noticed in several instances.

7) The 17-ketosteroid analyses must be regarded as an important help in arranging therapy with testosterone propionate for individual subjects.

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times that of women at the age of 20 to 40 years. In the oldest age groups the morbidity again decreases in both sexes.

The average height and weight of 75 diabetic men over 20 years of age were 171.2 cm and 73.5 kg. The corresponding values of 107 diabetic women were 157.7 cm and 68.1 kg. These figures may be compared to those found by *Schmidt* (1929), who measured 218 male and 215 Danish (psychiatric) patients. The average values found by *Schmidt* for height and weight were respectively 169.62 ± 0.48 cm and 67.38 ± 0.82 kg in men and 157.24 ± 0.41 cm and 60.20 ± 0.92 kg in women. Thus the height of adult diabetics did not significantly differ from the values found by *Schmidt*, while the weight was significantly higher. The weight distribution curve is asymmetric, deviating towards the overweight side, but showing only one maximum. The mean ages of *Schmidt's* patients were 43.6 and 47.6 years, while the mean ages in the present cases of diabetics were 49.5 and 54.6 years, in men and women respectively. This difference of age, however, only accounts for an increase of weight of 1 kg. at most, according to standard height and weight tables.

Admittedly, measurements on psychiatric patients cannot be considered identical with the values found in the normal population, but similar figures from Danish »normal patients« do not exist (measurements on recruits or on special classes of the population cannot be used).

Of the total of 102 men and 139 women, 68 per cent of the men and 74 per cent of the women were married or had been married previously. Of the general population in 1940, 48.9 per cent of the men and 53.3 per cent of the women were married or had been married previously. The difference between the diabetics and the general population with regard to marriage must be considered significant. An explanation of this is entirely lacking, but the fact that the age distribution of diabetics is different from that of the population as a whole must be taken into consideration (cf. *Joslin*, 1946).

The results given below are deduced from the 106 patients which were specially examined.

Sex glands.

With regard to sex function the most prominent symptom in the male diabetic is a lack of libido and a decrease in sexual potency. 17 of 19 patients questioned on this point showed these symptoms. It may be present even in well controlled diabetes, and once established it may continue in spite of adequate treatment of the diabetes.

20 diabetic men over 45 years had a total of 75 children or 3.8 each.

In women menstruation was as a rule not disturbed. Only one woman had had periods of amenorrhoea before the appearance of diabetes. Two had undergone radio-therapy for cancer of the uterine cervix. The menarche occurred at an average age of 14.6 years (50 cases), ranging from 12 to 20 years, while the menopause took place at an average age of 47.6 years (33 cases), ranging from 40 to 55 years. The values must be considered normal for Danish women.

In 30 of 61 women the diabetes appeared after the menopause. As seen from the diagram, there is no marked increase at the time of the menopause, the highest incidence being found later. Nor is there any marked increase of cases around the menarche.

34 women, who had passed the menopause at the time of examination, had borne a total of 154 children, or 4.5 each, and had 12 abortions. Only one of these 34 women had never given birth to a child.

Analysis of the urinary hormonal excretion was carried out in 28 men and 28 women (Table 2). The results were as follows:

The excretion of gonadotrophic hormones was within the normal limits in both sexes, being in nearly all cases lower than 50 m. u./24 hours. The excretion of oestrogen was also without any definite abnormality, though 9 out of 20 men excreted less than 20 m. u./24 hours. In most of the women the excretion of oestrogen was normal for the particular age. Only one, 34-years old, sexually mature woman, excreted no oestrogenic hormone. The patient had had a mild pulmonary

Table 2.
Urinary hormonal excretion per 24 hours in diabetics.

Men					Women			
Nr.	Age	gonado- trophin M.U.	oestro- gens M.U.	17-keto- stero- ids mg.	Age	gonado- trophin M.U.	oestrogens M.U.	17-keto- steroids mg.
1	4	50	0	0.5	4	<50	0	0.2
2	4	0	0	1.0	8	<50	<20	
3	13	<50	0	3.0	12	<50	ca. 10	4.5
4	16	<50	10	8.0	14	<50	ca. 10	3.0
5	23	50	50	6.5*	18	ca. 50	>20<200	6.5
6	30	<50	<20	3.5*	19	<50	ca. 100	6.0
7	34	<50	>20<50	15.0	27	ca. 50	>20<200	6.0
8	35	<50	ca. 10	1.5*	31	<50	ca. 100	
9	35	<50	>20<50	37.0	32	<50	>20<200	6.5
10	39	<50	ca. 10	5.0*	33	50	0	2.0*
11	39	0	0	8.5	39	<50	>20<200	3.5
12	40	0		3.0*	40	<50	>20<200	
13	40	<50	0	5.0*	43	>50	>20<200	
14	45	<50		8.5	47	<50	>20<200	
15	46	<50		9.0	56	50	ca. 20	3.0
16	47	<50			58	<50	>20<200	7.5
17	49	<50		5.5	60	<50	0	
18	49	<50	<20	3.5*	61	<50	ca. 10	3.0
19	51	<50	ca. 10	4.5*	63	<50	>20<200	3.5
20	55	<50	<20	9.5	63	<50	<20	3.0
21	59	<50		5.5	65	<50	<20	3.0
22	59	<50	ca. 50	7.0	67	<50	0	2.0
23	61	<50	<50	9.0	68	>50	<20	
24	62	<50	<50	3.5	68	<50	ca. 10	
25	63	ca. 50	<20	6.5	70	>50	0	4.0
26	66	<50	ca. 15	6.0	71	<50	0	
27	71	<50		5.2	72	<50	>20<100	4.0
28	76	<50	>20<50	6.5	73	<50	<20	2.5

Figures marked with an asterisk indicate a considerably decreased 17-ketosteroid excretion. Normal values: gonadotrophin < 50 M. U./24 hrs.; oestrogens (men) 20—50 M. U./24 hrs.; oestrogens (women) 20—200 M. U./24 hrs.; 17-ketosteroids see Fig. 2. The analyses have been carried out in the State Serum Institute, Copenhagen, under the supervision of C. Hamburger, M. D.

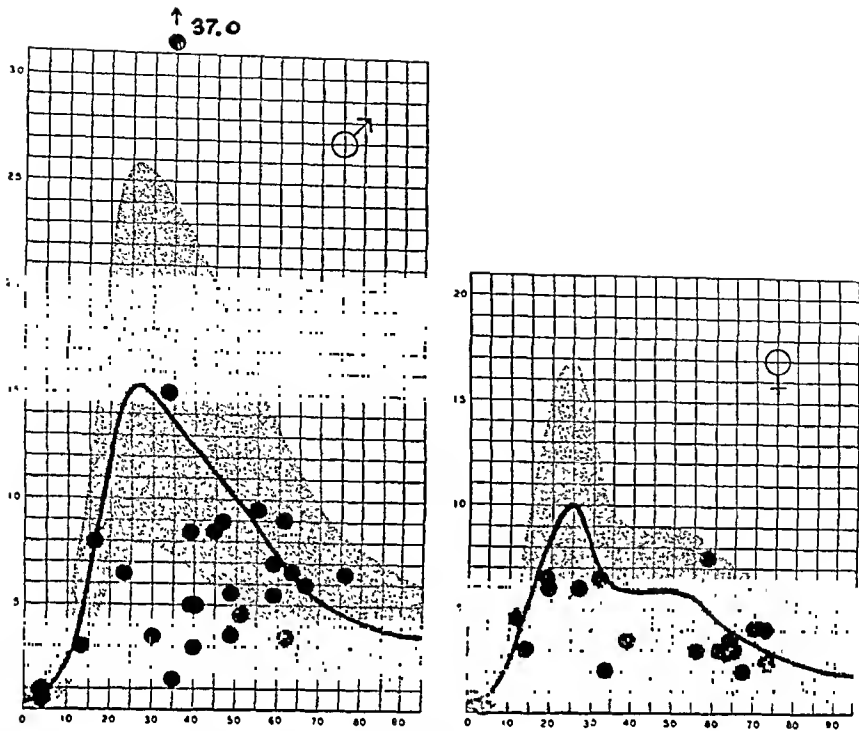


Fig. 2.

Excretion of 17-ketosteroids in 26 male and 19 female diabetic subjects. The black line shows the average output in normal subjects, and the stippled area gives the zone within which 97—98 per cent of normal values are falling, according to *Hamburger (1948)*.

Ordinates: mg/24-hours. Abscissae: age of the subject.

tuberculosis, but did not show any signs of hormonal dysfunction clinically.

The excretion of 17-ketosteroids was different in the two sexes (Fig. 2). Seven out of 26 diabetic men showed an excretion of 17-ketosteroids less than was found by *Hamburger (1948)*, in 97 per cent of normal men, though the same technique was used. All men with such abnormally low excretions were between the ages of 20 and 50. If only this particular age group is considered, then the deviation is still more marked, 7 of 13 subjects showing this low excretion, and 4 of the remainder being below the average. On the other hand, one 35-year old man showed an abnormally high excretion of 17-ketosteroids at the time immediately following a severe coma.

Further investigation of this case was impossible, as the patient was unwilling to continue his stay in the hospital.

In children, old people and in women, the excretion of 17-ketosteroids appeared within the normal limits, though not very high in the 4 middle-aged women in whom the analysis has been performed. The one woman with the abnormally low ketosteroid excretion was the same patient, who did not excrete any oestrogen.

Hypophysis.

X-ray examination of the sella turcica was performed in 40 men and 54 women. In no case was any definite enlargement found, nor were there any signs of other pathological changes, apart from partial or complete closure of the sella in four cases, due to calcification of the diaphragma sellae. In no case did the eye examination reveal any signs of intracranial tumour. No clinical signs of acromegaly were found in any case.

That the urinary gonadotrophin excretion was normal (i. e. not increased) in both sexes has already been mentioned.

Thyroid gland.

Enlargement of the thyroid gland was found in three cases, one case of exophthalmic goitre, and two cases with a non-toxic goitre. All these patients were women. Another woman was treated with methylthiouracil for hyperthyroidism; she had no goitre.

Adrenal glands.

In no case was any definite sign of adrenal disease found. The hair growth was normal, and there were no cases of marked hypertrichosis. »Bearded women« were found no more frequently among diabetics than in the general population. The 17-ketosteroid excretion is considered to be mainly of adrenal origin in women, and in children and old people of the male sex; as already stated the 17-ketosteroid excretion was in these groups within the normal limits.

Parathyroid glands.

Serum calcium determinations were carried out in 101 cases. The values varied from 8.3 mg per 100 ml. to 12.5 mg per 100 ml. with the majority around 10.0 mg per 100 ml. Seventy-one of the 101 values found were between 9.7 and 10.6 mg per 100 ml. The result does not suggest any abnormality of the parathyroid function in diabetes mellitus.

Basal metabolic rate.

The total basal metabolism was determined by the apparatus of *Krogh*. The determinations were carried out in the morning, after 14 hours' rest, and before giving food and insulin. No special diet was given, the patients being on the diet considered adequate in each case. A total of 272 determinations were made on 36 men and 47 women. The average basal metabolic rate was 109.6 ± 1.7 per cent in men, and 110.8 ± 1.6 per cent in women. The average basal metabolic rate is thus apparently slightly but significantly raised under these conditions.

These results, which are based on the oxygen consumption, are in complete agreement with those found by *Joslin* (1937) before the introduction of the undernutrition treatment (+ 12 per cent), and by *Holten* (1925) (+ 10.2 per cent).

Total serum cholesterol.

Determination of total cholesterol was carried out in 42 men and 59 women. The values varied from 42 to 470 mg per 100 ml. If, according to *Hunt* (1940) 230 mg per 100 ml. is considered the upper limit of the normal, 15 of 42 men and 30 of 59 women showed abnormally high values (36 and 51 per cent respectively). The percentages for men and women over 50 years were still higher, 44 and 59 per cent respectively.

DISCUSSION

The remarkably low urinary excretion of 17-ketosteroids, which is found in diabetic men of middle age, and to a lesser degree in women but not in children or in old people suggests

an inhibition of the function of the testes, since the 17-ketosteroid excretion in adult men is partly of testicular and partly of adrenal origin, while in women, children and old people it is mainly derived from the adrenals. Should further work support these results (and continued investigations have so far confirmed them), we may see in this finding an explanation of the impotence so common in diabetic men. In contrast to this, menstrual irregularities are nowadays uncommon in well controlled diabetic women. This is in agreement with the normal oestrogen excretion found in diabetic women.

The above results are in accordance with the findings of *Miller & Mason* (1945). These authors noted that diabetic patients of all ages excreted a smaller amount of 17-ketosteroids than do normal persons. The trend toward a lowered excretion was most marked in men but was also found in women.

White (1946) observed a tendency to higher 17-ketosteroid excretion in diabetic children than in normal controls.

While older statistics on sex incidence in diabetes invariably show the disease to be more common in men than in women (e. g. *v. Noorden*, 1917) this seems to be reversed in modern times. This trend has been noted in the United States by *Joslin* (1946), in Norway by *Hansson* (1947) and is also found in Sweden (*Dahlberg et al.*, 1947). *Joslin* believes that the reason for this is that women can more easily obtain adequate treatment, because of the higher appreciation of women in modern communities. Another explanation may be sought in the present increasing average duration of life. The high incidence of diabetes in women is particularly marked in the seventh decade, while the difference between men and women with regard to the incidence of diabetes is only slight during the first six decades as a whole.

The varying diabetic morbidity in women during life is another conspicuous fact. In childhood, the morbidity is the same in the two sexes, between 20 and 40 years the incidence in women is lower than in men, while the female sex is predominant among diabetics over 40 years, with a maximum in

the seventh decade. In still older people the morbidity decreases in both sexes. This peculiar distribution, which is practically the same as that found by *Joslin* (1937) in a very large number of cases, suggests the possibility that the female sex hormone protects to some extent against diabetes.

Some reports concerning the effects of oestrogens on diabetes have accumulated in the literature. Several workers report alleviation of menopausal diabetes in women following oestrogen treatment, with decreasing glycosuria and lower insulin requirement (*Cantilo*, 1941; *Gessler et al.*, 1939; *Gitlow & Kurschner*, 1943; *Mazer & Israel*, 1937; *Morton & McGavack*, 1946; *Schoene*, 1940; *Spiegelman*, 1933; *Thaddea & Hampe*, 1940). Negative results have also been reported (*Collens et al.*, 1936; *Kaufmann*, 1929; *Lawrence & Madders*, 1941). Conflicting evidence has also been obtained in animal experiments (*Barnes et al.*, 1933; *Ingle*, 1941 b; *Nelson & Overholser*, 1929; *Young*, 1941 a; *Zunz & La Barre*, 1939; *Foglia et al.*, 1947). The question cannot as yet be considered as decided.

A hypothetically abnormal pituitary activity might reveal itself in the gonadotrophic hormone excretion, enlargement of the sella turcica, growth abnormalities and in the basal metabolic rate. None of these findings have shown any definite signs of pituitary involvement in the disease. The sella turcica and the urinary gonadotrophic excretion was normal. The skeletal growth, as measured by the body height, was normal. The frequently increased height of the prediabetic child, described by *White* (1927), or the tendency to dwarfism sometimes noted in diabetes in children are not reflected in the average height of the usual diabetic.

The slightly raised basal metabolism is evidence that the condition is not similar to pituitary basophilism, since the basal metabolic rate is usually lowered in Cushing's disease. It might be compatible with a hyperactivity of the acidophile cells of the pituitary gland, but it could of course be explained in several other ways. Whether it is due to thyroid hyperactivity is unknown.

SUMMARY

A total of 107 non-selected consecutive cases of diabetes mellitus were examined with reference to the functions of the endocrine glands. The results were as follows:

The incidence in men was found to be approximately the same during the various stages of life, with the exception of a moderate increase in the sixth decade. In contrast to this, the incidence in sexually mature women is lower than in men of the same age, while in the more advanced years the incidence in women greatly surpasses that in men. This suggests that the female sex hormone may to some degree protect against diabetes.

The urinary excretion of 17-ketosteroids was extraordinary low in men between 20 and 50 years, low but within normal limits in women of the same age, and normal in children and old people. It is considered likely that the excretion of 17-ketosteroid of testicular origin is particularly decreased in male diabetics of middle age, thus giving an explanation for the impotence so common in these patients. The excretion of gonadotrophin and oestrogen was normal in both sexes.

No definite signs of abnormal pituitary, thyroid, parathyroid or adrenal activity were revealed.

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THE EFFECT OF SEX HORMONES IN SOME ORGANIC SOLVENTS, EMULSIFIED IN WATER

BY

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The introduction of a new dispensing form of the sex hormones needs justification by powerful arguments in its favour. The classical solution in oil is widely used and oil can be said to be a fairly satisfactory medium to which every physician is now accustomed. The main disadvantages are two-fold. Firstly, the difficulties inherent in the injection of oil, necessitating the use of rather thick needles, the discomfort the injection of oil may cause, and the difficulty of cleaning the syringe after use. Secondly a long lasting effect not obtainable with oil solutions is desirable in many cases. Improvements have been sought mainly for this reason.

The esterification of testosterone and oestradiol was a first, though not fully successful, step in this direction. Pellet implantation, introduced by *Deanesly & Parkes* (1937), though giving a satisfactorily prolonged action, calls for a minor operation with accompanying discomfort to the patient. Furthermore, the daily dosage is constant and cannot be modified should the need arise. The pellets may be expelled or capsule formation cause insufficient absorption. According to *Hartman* (1940) »the method is especially applicable where small amounts of hormone over long periods of time are desired«. Suspensions of crystalline hormones in water (*Freed & Greenhill*, 1941) have been investigated, with the impression

that the duration of effect of the hormone lies somewhere between that of the oil solution and of the implantation pellets. The duration of action is correlated to the size of the crystals (*Meier & Gasche*, 1946), but *Joel* (1948) considers that there is a limit to crystalline size because over a certain mesh injection of the suspension is painful. Control of crystal size is thus of importance. From our own experience we know that the crystals in such a suspension easily aggregate to larger clusters adhering to the walls of the container. These clusters may be so large as to clog the needle during injection, and in any case make the injection painful. These latter disadvantages of crystalline suspensions can be met to a large extent by their ad hoc preparation, as described by *Zondek & Rozin* (1948).

Wishing to go a step further our object has been to allow the crystals to form in the tissues rather than in the syringe. This effect can be obtained in the following way:

The addition of a sufficient amount of some inert material to the crystalline hormone has the effect of lowering the melting point of the crystals to room temperature so that a very strong solution is formed. This solution can be emulsified in water with the aid of detergents. If the added solvent has a much greater solubility in water than the hormone it will, after injection, rapidly dissolve in the tissue fluid leaving a deposit of the active agent.

The solvent used for lowering the melting point should have certain special properties, and the choice from among the many existing solvents is therefore considerably limited. It should be an extremely good solvent for the hormone. It should be almost immiscible with water, yet its solubility in water should be greater than that of the hormone. (Both these requirements exclude oil). It should be non-volatile (so as to be sterilisable by heat); it must be non-toxic. We have found benzyl alcohol well suited for this purpose. Most of the steroids dissolve extremely well in it though a few require added salol or camphor to lower the melting point of the mixture.

EXPERIMENTAL

This paper describes the results obtained with oestradiol monobenzoate and testosterone propionate.

Preparation of the emulsions.

50 gm. of testosterone propionate and 50 gm. of benzyl alcohol are mixed, the resulting solution shaken with a glycine buffer saturated with benzyl alcohol. The volume of the suspension should be 1 litre. Some »Tween 80« is added to stabilise the emulsion and glucose to make it isotonic. 10 gm. of oestradiol monobenzoate and 50 gm. of benzyl alcohol are mixed with 20 gm. of salol. The resulting solution is emulsified in the same way and to the same volume as in the case of testosterone propionate.

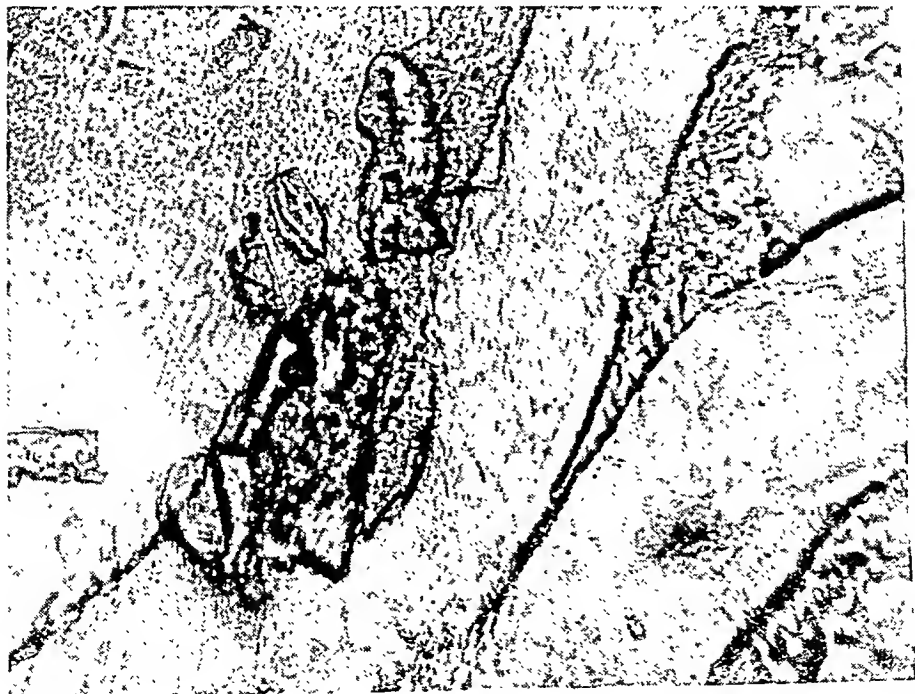


Fig. 4.

Crystals in the muscle of a rat, which had been injected with 0.2 ml of an emulsion of testosterone propionate on the previous day.

Properties of the emulsions.

The emulsions have a milky appearance. They can be sterilised by heat. If the emulsion breaks down after heating or prolonged standing it can be reconstituted simply by shaking the ampoule. When the emulsion is diluted with water the benzyl alcohol dissolves, leaving a suspension of crystals. As solubility of benzyl alcohol in water is about 40 mg. per ml., dilution of these emulsions with an equal volume of water or serum results in the formation of a crystal deposit. This occurs also *in vivo* and Fig. 1 shows crystals in the muscles of a rat which had been injected with 0.2 ml. of an emulsion of testosterone propionate on the previous day. There is, however, no risk of precipitation in a water-wet syringe so long as the amount of water present is not excessive.

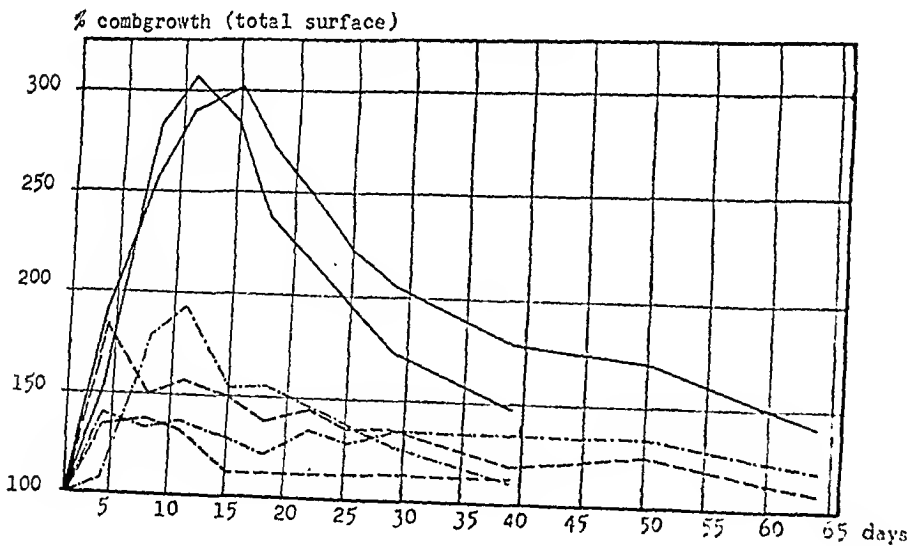


Fig. 2.

Combgrowth after one single intramuscular injection of 5 mg. testosterone propionate into capons. Each group consists of 6 animals.

— emulsion.

- - - oil solution.

..... crystal suspension prepared *ad hoc* in the 64 days test.

- · - · - commercial crystal suspension product in the 39 days test.

*Pharmacological activities.**a. Testosterone propionate.*

A comparison has been made of the biological activity of 5 mg. testosterone propionate, as dissolved in oil, as an emulsion and as a crystal suspension. The method used is the measurement of the growth of capons' combs after one intramuscular injection of each material. The results of two experiments each on groups of six animals are shown in Fig. 2. In the first experiment lasting 64 days the crystal suspension was prepared ad hoc. In the second experiment lasting only 39 days a commercial crystal suspension was used. The results show that the effect of the emulsion is much the greater and the more persistent. So great was the increase in the comb area as a result of the emulsion that it became impossible to measure with our photo-electric method, and the peak of the effect was greater than appears in the graph. The highest values quoted are understatements.

b. Oestradiol monobenzoate.

Similar experiments were performed in ovariectomized rats injected with oestradiol monobenzoate. The criterion was the duration of oestrus after subcutaneous injection of 0.5 mg. of the substance as dissolved in oil, as an emulsion and as a crystal suspension. A crystal suspension of oestradiol and an emulsion of diethylstilboestrol were also compared. A group of 6 animals was considered to react positively when 67 per cent showed oestrus. The results are summarized in Table 1. A first experiment using the emulsion and a crystal suspension of our own manufacture, both of oestradiol benzoate, caused a much more prolonged oestrus than either the oil solution of oestradiol benzoate or the crystal suspension of oestradiol. In a second experiment in which a commercial crystal suspension was used, the activity of both the emulsion and the crystal suspension again proved to be much better than that of the oily solution. These experiments were complicated by the

Table 1.

Nr. of experiment	Preparation	Nr. of animals	Duration of oestrus in days	Remarks
I	emulsion	6	12	of own manufacture
	crystal suspension	6	12	
	solution of oil	6	5	
	oestradiol crystals	6	6	
II	emulsion	12	7	commercial product
	crystal suspension	12	4	
	solution in oil	11	4	
	diethylstilboestrol —			
	emulsion	11	3	

The oestrogenic effect of oestradiol monobenzoate in different media in castrated rats. Total dose in all series 0.5 mg. As a comparison oestradiol crystals are included.

fact that not all the animals in the groups treated with the emulsion and the crystal suspension attained a full anoestrus. They remained in met- or pro-oestrus or even attained a renewed state of full oestrus. This was not the case with the animals treated with the oily solution or with the stilboestrol emulsion. This peculiar phenomenon will be the subject of further investigations.

c. Toxicity.

Toxicity of the emulsions was studied in rats, dogs, cats, frogs, rabbits and man. Experiments were performed with 2 types of emulsion, one with and the other without salol.

Groups of 4 male and 4 female rats with an initial body weight of 35—40 gm. were injected subcutaneously with 0.25 ml. of the emulsion, without hormones, daily for a period of 6 weeks. A third group received 0.09 per cent of a saline solution. The average weight increase in the groups was as follows:

Saline injected — 90 gm. — none dead.

Emulsion with salol — 80 gm. — one dead

Emulsion without salol — 85 gm. — two dead.

The differences in weight of the three groups are probably insignificant. A few deaths occurred after about 4 weeks of treatment, and are probably accidental, the other animals remaining in perfect condition. No local effects were observed, except slight hardening of the skin at the site of injection.

One dog was injected intramuscularly with 1 ml. of the emulsion containing salol, and another dog with 1 ml. of the emulsion without salol. No local or general reaction was observed.

Injection of 0.5 ml. into the ventral lymph sac of frogs caused the death of all the animals within 15 minutes. 0.2 ml. caused death in all the frogs injected with the emulsion containing salol and in 3 out of 5 frogs injected with emulsion without salol. 0.1 ml. of both emulsions was tolerated by two groups of 5 animals. All the frogs used were rather small, their body weight varying from 10 to 30 gm.

The local effect was studied by sub-conjunctival injection (6 animals) or injection into the anterior chamber of the rabbit's eye (2 animals). Some swelling and hyperaemia occurred, but were not more marked than after similar injection of innocuous solutions. The effect was transitory, maximal reaction occurring after about 6 hours. Hardly any reaction could be observed after 24 hours and the effect had entirely vanished after 48 hours. The emulsions containing salol caused the same reactions as those without salol.

Finally, 1 ml. was injected intramuscularly into 6 men. No pain and no local or general reactions were observed.

DISCUSSION

These experiments show that in animals the activity of testosterone propionate and oestradiol monobenzoate as emulsions is definitely not lower and probably higher than that of crystal suspensions of the same substance, and the effect of the emulsions is superior to that of the corresponding solutions in oil. These results warrant clinical experiments with these emulsions, particularly because their administration offers technical advantages over both crystal suspensions and oil solutions. From the toxicity experiments it is clear that a daily dose of 2.5 to 5.0 ml. per kg. is not toxic in rats in experiments lasting 6 weeks. The same dose per kg. is not toxic in frogs either. The amount of emulsion administered to patients would normally be as little as 1 ml.; larger volumes will seldom be required. There is no reason to fear any untoward effects whatever from such an injection. No increase in the duration of action of diethylstilboestrol results from its administration in the form of an emulsion. The reason for this is still obscure. The differences in the effects of suspensions of oestradiol and oestradiol monobenzoate bear a superficial resemblance to the differences between the corresponding oil solutions, but the mechanism involved may be quite different.

The principle used in the preparation of these emulsions can be extended to other steroid hormones, such as desoxycorticosterone acetate and progesterone. Experiments with these substances are in progress, and the results will be reported in due course.

SUMMARY

Emulsions of testosterone propionate and oestradiol benzoate have been prepared from their solutions in benzyl alcohol. In animal experiments these emulsions produce effects comparable to or even better than those of crystal suspensions. Effects having a much greater duration are obtained with these

emulsions than with solutions in oil, and the disadvantages of the administration of oil solutions are avoided.

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